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CHARACTERIZATION OF A TRANSCRIPTIONAL ACTIVATOR IN THE ENVELOPE GENE OF MOUSE MAMMARY TUMOR VIRUS

BY

CINDY L. MILLER

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF THE DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA

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ABSTRACT

Mouse Mammary Tumor Virus (MMTV) is the causative agent of mammary carcinomas in certain mouse strains. There is also evidence indicating that it may be associated with the development of T lymphomas. Transcription of the complete MMTV proviral genome in mouse cells is controlled by a strong promoter in its long terminal repeat (LTR). In the mouse T lymphoma cell line EL4.E1, there is a second, activation-dependent, transcriptional activator sequence within the MMTV envelope (*env*) gene. Phorbol ester treatment of EL4.E1 cells generates a transcript initiating within the *env* gene, which includes the open reading frame gene of the 3' LTR.

I have isolated and characterized a segment of the MMTV *env* gene (called META for MMTV *env* transcriptional activator). META was linked to the chloramphenicol acetyltransferase (CAT) gene for use in transient-expression assays. META induced activation-dependent, T lymphocyte-specific expression of the CAT gene. It was active in mouse and human T helper cell lines but not in other cell types. META activity was dependent on activation of the T helper cell line with the same stimuli which induced cytokine production and its activity was suppressible by the immunosuppressive drug, Cyclosporin A. META has been isolated from EL4.E1 cells, from a T cell hybridoma, and from BALB/c spleen cells. It was also demonstrated that a portion of META acts as an inducible, orientation-independent, CsA-sensitive enhancer when linked to a heterologous promoter. A model for the potential involvement of META in MMTV-induced T lymphomagenesis is presented.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. V. Paetkau for his support and guidance throughout my graduate studies. I would also like to acknowledge the support of my supervisory committee and other members of the Department of Biochemistry including Dr. C. Spencer, Dr. C. Bleackley, Dr. L. Guilbert, Dr. J. Weiner and Dr. W. Paranchych.

It was a pleasure to be associated with the Department of Biochemistry during the time spent in graduate studies. There are many individuals who taught me a great deal and were always willing to be of assistance. Their friendship and support provided both a stimulating working environment and enjoyable social occasions.

Poppi Havele and Jonathan Hooton freely shared their tissue culture experience and Jennifer Shaw, Bill Pohajdak and Dale Talbot introduced me to the wonders of molecular biology. Special thanks is extended to Rosemary Garner and Cliff Gibbs who provided excellent technical assistance. Rosemary Garner and Jonathan Hooton were collaborators on several studies presented in this thesis. Roger Bradley and Cliff Gibbs did the necessary photographic work. I would also like to acknowledge the contributions made by members of the Paetkau and Bleackley laboratories including Ella Schreiber, Farzana Esmail, Jaling Esmail, Padma Mandalpu, Nancy Ehrman, Brenda Duggan, Cheryl Helgason, Chantal Fregeau, Eric Atkinson, Irene Shostak, Ole Sorenson and John Prendergast, .

A special dedication is extended to my daughter, Michelle and members of Litchfield and Miller families. Their continued interest and concern was very important to me during my graduate studies.

I would like to acknowledge the financial support of the Alberta Heritage Foundation for Medical Research.

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LIST OF ABBREVIATIONS

acetyl CoA	acetyl coenzyme A
AP-1	Activator protein-1
APC	Antigen presenting cell
bp	Base pairs
BSA	Bovine serum albumin
CAT	Chloramphenicol acetyltransferase
cDNA	Complementary DNA
CD	Cluster of differentiation
Con A	Concanavalin A
CP	Core protein
CsA	Cyclosporin A
CsCl	Cesium chloride
CTL	Cytotoxic T lymphocytes
DEAE-dextran	Diethylaminoethyl-Dextran
DMBA-LV	Dimethylbenzanthracene leukemogenesis virus
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetracetic acid
env	envelope
FKBP	FK-506 binding protein
GAG	glycosaminoglycans
GM-CSF	Granulocyte macrophage colony stimulating factor
GRE	Glucocorticoid response elements
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
ICAM-I	Intracellular adhesion molecule I
IFNγ	Interferon gamma
IL2Rα	Interleukin 2 receptor α
IL	Interleukin, such as Interleukin 2
IN	Integrase
kDa	Kilodaltons
LB	Luria-Bertani
LFA1, LFA3	lymphocyte function associated antigen
LPS	Lipopolysaccharide

LTR	Long terminal repeat
MCS	Multiple cloning site
META	MMTV *env* transcriptional activator
MHC	Major histocompatibility complex
Mls	Minor lymphocyte stimulating
MMTV	Mouse Mamary Tumor Virus
MoLV	Moloney leukemia virus
MOPS	3-[N-morpholino] propanesulfonic acid
mRNA	Messenger RNA
NF-AT	Nuclear factor of activated T cells
NF	Nuclear factor
NFκB	Nuclear factor kappa B
NRE	negative regulatory elements
nt	Nucleotide
orf	Open reading frame
PB	Primer binding
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGCP	proteoglycan core protein
PIPES	Sodium salt of piperazine N,N-bis{2-ethanesulfonic acid}]
PKC	Protein kinase C
PMA	Phorbol-12-myristate-13-acetate
pol	polymerase
PPIase	Peptidyl-prolyl cis trans isomerases
pro	protease
RNase	Ribonuclease
rRNasin	Recombinant ribonuclease inhibitor
RSV	Rous Sarcoma Virus
SDS	Sodium dodecyl sulfate
SV40	Simian sarcoma virus 40
TBS	Tris-buffered saline
TCEd	T cell element, distal
TCEp	T cell element, proximal
TcR	T cell antigen receptor
TGF-β	Tranforming growth factor-beta

TLC	thin layer chromatography
TNFα	tumor necrosis factor-alpha
TPA	12-O-tetradecanoyl phorbol-13-acetate (PMA)
TREd	TPA responsive element, distal
TREp	TPA responsive element, proximal
tRNA-lys	Transfer RNA-lysine
UCR	Upstream conserved region
V	Variable

CHAPTER ONE. INTRODUCTION

Overview of the immune system

The immune system has evolved to provide mechanisms by which bacteria, viruses and other potential pathogens can be eliminated. An immune response involves the participation of many distinct cell types whose functions must be coordinated to provide a specific response of appropriate magnitude and duration.

Adaptive immunity is necessary for an animal to discriminate between self and non-self antigens (reviewed in 15, 157, 205). It begins early in development, when somatic rearrangement of the genes encoding the T cell antigen receptor (TcR) gives rise to receptors with the potential of recognizing a wide variety of antigenic determinants. Cells bearing TcR which bind very strongly to modified major histocompatibility complex (MHC) antigens present in the developing thymus are eliminated (negative selection), thereby preventing autoreactivity. Those which have no affinity for available antigens probably are lost due to lack of stimulation. But thymocytes which bind self antigens with a moderate affinity survive and mature (positive selection), and form the basis of the T lymphocyte immune repertoire.

T lymphocytes play a central role in the regulation of immune responses. These cells can be broadly classified into two major groups based on both their function and the phenotypic markers. These markers, known as clusters of differentiation (CD), are present on cell surfaces. During their development, thymocytes bearing both CD4 and CD8 surface markers differentiate to either single-positive $CD4^+$ or $CD8^+$, mature, T lymphocytes.

$CD8^+$ T lymphocytes include cytotoxic T lymphocytes (CTL), which destroy cells presenting unusual antigens on their surfaces. These antigens may result from viral infection, transformation of the cell to a malignant phenotype, or from organ transplantation. The isolation and characterization of another $CD8^+$ cell, the antigen-specific T suppressor lymphocyte, has been difficult. However, these cells are thought to play a role by down-regulating immune responses.

The second major subset of T lymphocytes are $CD4^+$ T helper cells. Their role is to amplify a specific immune response by secretion of factors,

known as cytokines, which act in autocrine or paracrine manner to support the maturation and proliferation of T cells, B cells and other hemopoietic cells. Cloned murine T helper cells have also been further divided into two groups based on their pattern of cytokine release (141). T_{H1} cells secrete a set of cytokines including Interferon γ (IFNγ), Granulocyte Macrophage Colony Stimulating Factor (GM-CSF), Interleukin 2 (IL2) and lymphotoxin. IL4, IL5, IL6 and GM-CSF are among the factors produced by T_{H2} cells. This clear subdivision breaks down into various other sets upon closer analysis however.

Activation of T cells

The activation of T lymphocytes is discussed in detail in a number of good review articles (35, 38, 109, 173). In general, mature T lymphocytes recognize peptides associated with the MHC on the surfaces of various cells. MHC molecules can be divided into two classes: Class I molecules present peptides which arise from proteins synthesized within the cell, while Class II molecules display peptides which result from the breakdown of exogenous proteins. CTL recognize peptides presented in association with Class I molecules, while mature T helper cells recognize peptide antigens bound with MHC Class II. Developmentally-mature, quiescent T helper lymphocytes recognize antigens in the context of MHC Class II molecules on the surfaces of special antigen-presenting cells (APC), such as B lymphocytes, activated macrophages, or dendritic cells.

The TcR is a disulfide-linked heterodimer usually comprising one chain each of products of the α and β TcR gene families. The V (variable) and other elements of the α and β genes are rearranged during ontogeny to give rise to a functional, expressible receptor chain. Associated with the TcR are the invariant, noncovalently linked subunits of the CD3 complex (208). These include the gamma, delta, and epsilon chains which are all integral membrane proteins forming the core of the CD3 complex. The zeta chain contains a nucleotide binding site which may be involved in signal transduction, and associates with the complex as either a homodimer, or as a heterodimer with a truncated form of zeta known as nu. It is thought that zeta-zeta or zeta-nu dimers are coupled to different signal transduction pathways. In contrast to the subunits of the TcR, the invariant components of the CD3 complex have large cytoplasmic domains which are thought to couple the TcR to signal transduction pathways.

Association between the TcR and antigens presented in the context of MHC on the appropriate cell is the first step in the activation of a T cell. This association is enhanced by binding of the CD4 (or CD8) molecule to a site on the MHC molecule which is distinct from that recognized by the TcR. These interactions initiate a series of events which lead to the production of second messengers within the T cell. There appear to be two major pathways involved in the generation of second messengers. The first involves the activation of phospholipase C, which cleaves phosphatidylinositol 4,5-bisphosphate into 1,2-diacylglycerol and inositol 1,4,5-trisphosphate. In turn, diacylglycerol activates protein kinase C (PKC), while the inositol trisphosphate increases the concentration of intracellular calcium by inducing influx through calcium channels (71). Activation of T cells, including induction of cytokine gene transcription, can often be achieved with a combination of ionomycin and phorbol 12-myristate 13-acetate (PMA), agents that increase intracellular Ca^{++} concentrations and activate protein kinase C, respectively (94, 195).

The second pathway, which has not been as well characterized in T lymphocytes, involves the activation of one or more protein tyrosine kinases (110). For example, it is thought that $p59^{fyn}$ may be involved in the phosphorylations observed immediately following TcR stimulation. Another kinase which may be involved in the phosphorylation of the zeta chain is $p56^{lck}$. It has been shown to associate with the cytoplasmic tails of both CD4 and CD8 antigens. Leukocyte common antigen, CD45, shown to have tyrosine phosphatase activity, appears to be important for the coupling of TcR signaling to the phosphatidyl inositol pathway. This coupling may be mediated by the dephosphorylation of Tyr 505 of $p56^{lck}$ by CD45, thereby enhancing the activity of the kinase (67). Regardless of the signal transduction pathway utilized in initial activation of mature T lymphocytes, a secondary stimulation to induce cell division is also required. In many instances, this signal is provided by the interaction of IL2 with its receptor.

The generation of cytokines by $CD4^+$ T helper lymphocytes is initiated by TcR-dependent pathways. It appears that a costimulatory signal is also required to augment activation. Several types of costimulatory mechanisms have been characterized. The first involves a functional or physical interaction between other sets of receptors, which results in the enhancement of the original signal transduction pathway. An example of this is the interac-

tion between LFA3 and CD2. Secondly, cell adhesion molecules such as LFA1 and ICAM1 can increase the avidity of the interaction between the T cell and the APC. Finally, a receptor-ligand interaction may have the capacity to initiate signaling in isolation, but can also synergize with signals resulting from engagement of the TcR. Such may be the case with CD28.

The CD28 molecule is present on the surface of most mature T cells. Treatment with anti-CD28 antibodies in combination with antigen or agents which mimic TcR-mediated activation results in the augmentation of mRNA levels for IL2, TNF-α, GM-CSF, IFNγ and lymphotoxin (101, 102, 189). Increases in IL2 mRNA are due to both an enhancement of gene transcription and by stabilization of the mature mRNA (118). Fraser and coworkers have identified an element present within the IL2 enhancer (-140 to -164) that is the target for a CD28-regulated nuclear binding complex (62).

The ligand for the CD28 receptor is the B7/BB1 antigen present on activated B cells (122a). B7-transfected Chinese hamster ovary cells can increase the proliferation and IL2 gene expression of cocultured, suboptimally activated $CD28^+$ T cells, suggesting that interaction of B7 with CD28 can generate a costimulatory signal capable of amplifying IL2 gene expression (122). Dual signaling, mediated by the antigen-TcR interaction and by CD28- ligand binding may also be an important mechanism to prevent induction of clonal anergy (83a).

Activation Induced Programmed Cell Death

In some instances stimulation of the TcR leads to cell death. This activation-induced, programmed cell death or apoptosis (reviewed in 33) is characterized by chromatin condensation, fragmentation of cellular DNA into nucleosome size fragments by an endogenous nuclease, and blebbing of the cytoplasm. Phagocytosis of the membrane-bound components of the dying cell may prevent the elicitation of an inflammatory response. Apoptosis is thought to be an important mechanism in the negative selection of autoreactive thymocytes during the establishment of tolerance. Murphy and coworkers generated transgenic mice expressing a TcR recognizing chicken ovalbumin. Administration of peptide antigen resulted in apoptosis in the thymic cortex and rapid deletion of immature CD4+CD8+ cells, providing evidence for the role of activation-induced programmed cell death in thymic selection (143).

Apoptosis has been shown to occur in T cell lines and T cell hybridomas activated through the TcR by antigen or by anti-CD3 (179, 180) and in hematopoietic cells deprived of colony stimulating factors (210). The immunosuppressive agents CsA and FK506 (discussed in the following section) prevent apoptosis in activated T cell lines if added immediately following the inductive signal suggesting that the compounds interfere with the calcium dependent events involved in fragmentation of the genomic DNA (180, 185).

Regulation of IL2 gene expression

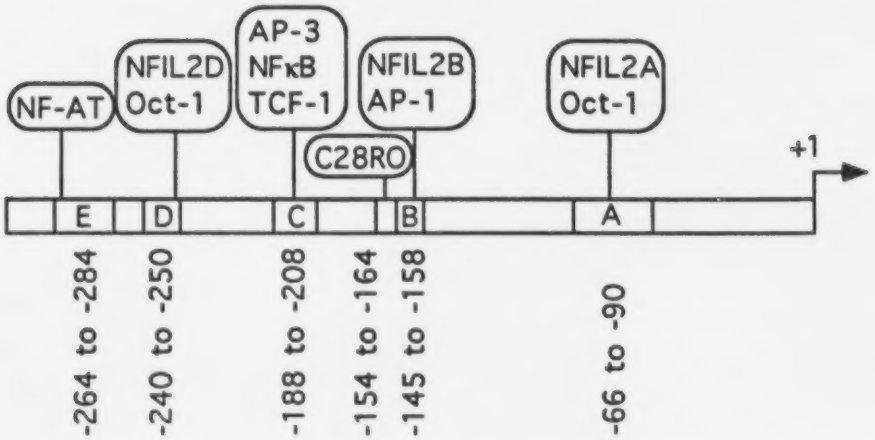
Activation of certain T helper cells by either antigen or treatment with pharmacological agents which mimic antigenic stimulation leads to the induction of IL2 gene expression (reviewed in 200). IL2 mRNA is detectable within 1 to 6 hours following activation of T cell lines and human peripheral blood lymphocytes, and has a half life of about 1 hour (176). There are several mechanisms controlling the level of IL2 mRNA, including the frequency of transcription initiation, rate of transcription, and stability of the mRNA.

Studies have localized an activation-dependent enhancer in the 5' flanking regions of the IL2 gene within sequences -326 to -52 nucleotides from the start of transcription (66, 174, 200). Several distinct regions have been mapped to which nuclear factors bind following activation of T cells (Figure 1-1). Mutation of one or more of these sites results in a decrease in IL2 gene expression. These regions include binding sites for constitutively expressed proteins such as Oct-1 and AP-3, and inducible binding proteins such as nuclear factor of activated T cells (NF-AT), NFκB, and AP-1.

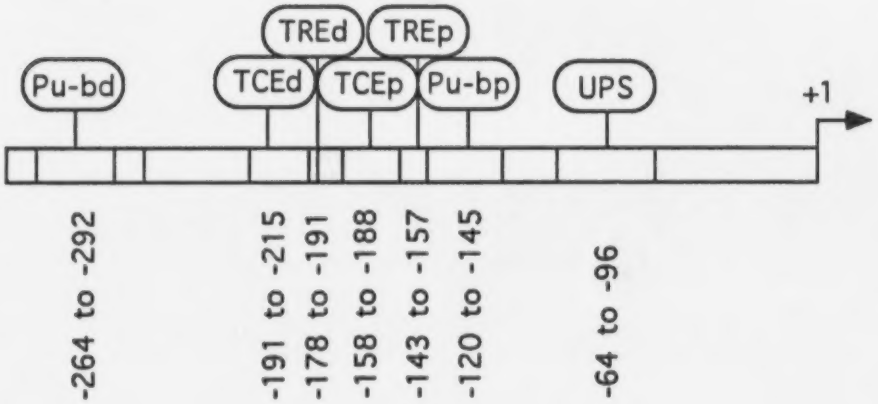
In the mouse IL2 gene, AP-1 sites are present within the distal and proximal TPA response elements, TREd and TREp. No binding at the distal site in the murine IL2 gene (-180) was detectable by footprinting analysis (96), and deletion of this site did not affect induction of the IL2 promoter (97). Binding to the proximal site (-150) has been detected using extracts from activated T cells. Nuclear extracts prepared from cells depleted of PKC showed a decrease in binding activity, suggesting that PKC induction of AP-1 is important for expression of the IL2 enhancer. Deletion of the NFIL2B site in the human IL2 promoter reduces its activity to about 11% of the wild-type level

Figure 1-1 Known binding sites of the mouse and human IL2 enhancer
The numbers indicate the nucleotide boundaries of the protein binding sites in the mouse (18) and human (47,62) IL2 enhancer. Some of the transcriptional regulatory proteins thought to bind to the human IL2 enhancer are indicated and described further in the text. For the mouse IL2 enhancer the designation of the protein binding motifs are as follows, Pu-bd - distal purine box; Pu-bp-proximal purine box; TCEd-distal T cell element; TCEp-proximal T cell element; TREd-distal TPA responsive element; TREp-proximal TPA responsive element; UPS-upstream promoter site.

Human IL2 promoter/enhancer



Mouse IL2 promoter/enhancer



in Jurkat cells (47) and mutations of the Ap-1 site within it decreases enhancer activity by as much as 95% (97).

Two elements in the murine IL2 enhancer, designated, TCEp and TCEd, were protected in DNase I footprinting assays using extracts from TPA-stimulated EL4 cells (174). These sites bound a factor indistinguishable from the AP-3 purified from HeLa cells. The TCEd has been shown to bind a factor called TCF-1 (T cell factor 1) which consists of two polypeptides. One subunit may be related to the 50 kDa polypeptide of NFκB (20). It has been suggested that a homodimeric complex of NFκB p50 subunit, bound to the IL2 enhancer, acts as a repressor in quiescent T cells and is displaced by NFκB p50-p65 complexes following antigenic stimulation (103).

Binding of Oct-1 to the IL2 enhancer may be important for the regulation of IL2 transcription. Mutation of the Oct-1 binding site within multimers of NFIL2A reduces CAT activity in activated Jurkat cells by about 70% (199). Interaction of Oct-1 with its target sequence involves the participation of an inducible associated protein (OAP40). The importance of the distal Oct-1 site in IL2 gene regulation has not been established.

NF-AT appears to be a critically-important transcriptional regulatory complex required for IL2 gene expression (18, 178). The NF-AT complex binds to purine-rich regions located in the mouse and human gene at -264 to -292 and -264 to -284 respectively. Binding of nuclear proteins to these sites occurs 10 to 25 min prior to the start of IL2 gene transcription. Binding is not detected in uninduced cells or in cells treated with protein synthesis inhibitors prior to activation, indicating that formation of a functional NF-AT complex requires new protein synthesis. Flanagan and coworkers have provided evidence that a pre-existing cytoplasmic subunit of NF-AT translocates to the nucleus in response to signaling from the antigen receptor. Its combination with a newly-synthesized nuclear subunit may be required to mediate transcriptional enhancement of the IL2 promoter (58). There is evidence that the Fos-Jun AP-1 complex may form the inducible nuclear component of NF-AT (95).

Effects of Cyclosporin A, FK506 and Rapamycin on T cell function

Cyclosporin A (CsA) and FK506 are potent immunosuppressive drugs widely used to prevent rejection in patients receiving organ allografts. They are fungal metabolites isolated from *Tolypocladium inflatum* and

Streptomyces tsukubaensis, respectively (reviewed in 181, 191). Although structurally unrelated, evidence indicates that both compounds alter specific Ca^{++}-dependent events in T-cells (7, 181). Both immunosuppressants affect components of signal transduction pathways involved in the regulation of cytokine gene expression, initiation of activation-induced programmed cell death and exocytosis. FK506 is more potent than CsA in inhibition of T cell function, with an ED_{50} of 0.2-0.5 nM compared to 10-100 nM for the latter. Rapamycin, isolated from *Streptomyces hygrosopicus*, is structurally related to FK506 but appears to inhibit T cell function by a mechanism distinct from either CsA or FK506.

CsA and FK506 block T cell proliferation induced by lectins such as Con A, by monoclonal antibodies to the TcR or to the CD3 complex, and by a combination of calcium ionophores and PMA (46, 106, 130, 193). FK506 and CsA also inhibit T cell proliferation in response to cytokines such as IL2 and IL4, but only at high doses. There are reports indicating that proliferation in T cells in response to treatment with anti-CD28 and PMA is resistant to FK506 (105) and CsA (101) but sensitive to Rapamycin (13). In contrast, Rapamycin interferes with T cell proliferation stimulated by cytokines such as IL2, IL4 and IL6 (13, 45). The expression of IL2 receptor chain α is not inhibited by CsA or FK506 (159, 193).

CsA has been shown to inhibit the death of thymocytes in animals treated with anti-CD3 (179). In vivo administration of CsA prevents the generation of single positive thymocytes (either $CD4^+CD8^-$ or $CD4^-CD8^+$) and interferes with the deletion of cells bearing self-reactive TcR (70, 98, 168). Inefficient deletion of these autoreactive clones by inhibition of apoptosis may result in the emergence of cells capable of causing autoimmune disease. The decrease in the development of mature thymocytes may be due both to a lack of lymphokine production required for T cell expansion and a toxic effect of CsA on thymic cells expressing MHC Class 11 which are required for positive selection (70).

One, if not the principal, way in which CsA and FK506 suppress T cell function is by inhibiting the expression of a set of cytokine genes including IL2, IL3, IL4, IL5, GM-CSF, tumor necrosis factor alpha (TNFα) and IFNγ (50, 77, 160, 193). Rapamycin has little effect on lymphokine mRNA synthesis or secretion (193).

CsA has also been shown to inhibit the release of granule-associated serine esterases from murine CTL triggered with either anti-CD3 or calcium ionophore plus PMA resulting in a partial inhibition of lytic activity (116). CsA also blocks the generation of functional CTL in a mixed lymphocyte response although it does not inhibit the development of a population of precursor CTL which require only IL2 to become cytotoxic (92).

Effect of CsA and FK506 on IL2 Expression

The most extensively studied system of inhibition of lymphokine gene expression by FK506 and CsA is that of the IL2 gene. As discussed, induction of the IL2 gene is regulated primarily by a region which extends about 300 bp upstream of the transcription start site. Nuclear run-off experiments indicate that both compounds interfere at the level of gene transcription. Two regions of the IL2 enhancer that appear to be the most sensitive to CsA and FK506 are the binding of NF-AT to the NFIL-2E site and Oct-1 binding to NFIL-2A.

Investigators have examined the effects of the immunosuppressants on various elements present in the IL2 promoter using tandem repeats of the element linked to a basal promoter placed upstream of a reporter gene such as Chloramphenicol acetyl transferase. The induced transcription driven by the promoter elements NFIL2E (NF-AT binding site) and NFIL2A are completely blocked by FK506 and CsA (10, 52, 78). The induced activity from multimers of either the NFκB site or the AP-3 site are partially suppressible by both agents (80, 127). CAT activity driven by a basal promoter and multimers of an AP-1 oligonucleotide in induced T cells has been reported to be either partially inhibited (78) or resistant to both drugs (52).

It has been proposed that both CsA and FK506 interfere with Ca^{++}-dependent signal transduction pathways involved in the activation of the NF-AT binding complex. There is evidence that CsA inhibits the translocation into the nucleus of a preformed cytoplasmic subunit of NF-AT (39). Neither cytoplasmic nor nuclear extracts of activated T cells treated with CsA and FK506, contain binding activity to the NF-AT binding site. Binding activity can be reconstituted by mixing nuclear extracts from stimulated, drug-treated cells with cytosolic extracts from either non-stimulated or immunosuppressant-treated cells (58). The Fos-Jun AP-1 complex may form the inducible, CsA- and FK506-insensitive nuclear component of NF-AT.

Jain and coworkers found that NF-AT binding activity was reduced if nuclear extracts prepared from activated T cells were first depleted of Fos protein. Binding activity could be restored by the addition of nuclear extracts from stimulated CsA-treated T cells but not with nuclear extracts from unstimulated cells (95). Taken together these results are consistent with a model in which a CsA-sensitive cytoplasmic factor is translocated to the nucleus, where it combines with a newly synthesized nuclear subunit, possibly AP-1, to form an active NF-AT transcriptional regulatory complex.

Immunophilins

CsA, FK506 and Rapamycin bind with high affinity to cytoplasmic proteins termed immunophilins (172). Immunophilins are of two classes, the cyclophilins, which bind to CsA (82), and the FK506 binding proteins (FKBPs) which bind to FK506 and Rapamycin (84). Both of these classes of proteins catalyze the cis - trans isomerization of a peptidyl-prolyl bond in peptide and protein substrates, and therefore are termed peptidyl-prolyl cis - trans isomerases (PPIase). The rotamase activities of the immunophilins are strongly inhibited by their respective immunosuppressant ligands.

Both cyclophilins and FKBPs have been identified in yeast, bacteria and in different tissues of higher eukaryotes. The function of the immunophilins is unclear, but they presumably catalyze protein folding. The *nina A* gene of *Drosophila* encodes a protein similar to cyclophilin and it appears to be important for trafficking one form of rhodopsin to the membrane. A member of the FKBP family, FKBP 59, in association with heat shock proteins hsp70 and hsp90, is complexed with the glucocorticoid receptor to form an inactive steroid hormone complex (187). FKBP 59 may be involved both in the assembly of the inactive complex and dissociation of it upon binding of steroid hormones. Further characterization of the immunophilins and associated proteins in vivo is required to delineate their physiological function.

As discussed, CsA and FK506 are both potent inhibitors of IL2 gene transcription in activated T cells. It has been hypothesized that the interaction of immunophilins with their drug ligands inhibits their PPIase activity. Inhibition of protein folding may decrease the ability of the transcriptional factors to bind DNA or carry out transcriptional activation. Several lines of evidence make this model unlikely. Rapamycin blocks the rotamase activity

of FKBP, but inhibition does not result in the suppression of IL2 gene transcription. An analog of FK506, FK506BD, also binds FKBP and inhibits its isomerase activity without interfering with T cell activation. Both compounds can compete with the binding of FK506 to FKBP and inhibit its activity in T cells. It has also been shown that the concentration of the immunosuppressants necessary for inhibition of IL2 gene expression is lower than that required to saturate the intracellular levels of the corresponding immunophilin (reviewed in 181).

These observations and others have led to an alternate hypothesis in which binding of drug to immunophilin results in a gain-of-function (172). In this model, additional protein(s) may associate with the immunophilin-immunosuppressant complex. This binding may inhibit the function of the third component, resulting in suppression of T cell activation and IL2 transcription. A potential target for the immunophilin-immunosuppressant complex is a calmodulin-dependent phosphatase, calcineurin (123). Liu and coworkers made affinity columns of FKPB12-FK506 or Cyclophilin C-CsA (64) to purify a number of proteins from calf thymus extract. These proteins were not eluted with free immunophilins or unbound drugs, or in the case of the FKPB12-FK506 matrix, with the FKBP12-Rapamycin complex. The proteins obtained were identified as calmodulin and two subunits of calcineurin. It has also been shown that the phosphatase activity of calcineurin is inhibited the drug-immunophilin complexes in vitro (65, 123).

It has recently been demonstrated that overexpression of calcineurin (31) or its catalytic subunit (148) in Jurkat cells renders them more resistant to the affects of CsA and FK506. Additionally, the increased levels of calcineurin facilitate transcription dependent on the IL2 promoter, NF-AT, or NFIL2A in the presence of sub-optimal concentrations of ionomycin. It has been proposed that calcineurin may regulate the translocation of the cytoplasmic component of NF-AT into the nucleus. It may also be involved in the formation of a functional OAP/Oct-1 complex. By this mechanism, inhibition of calcineurin activity by the drug-immunophilin complex would interfere with the Ca^{++}-dependent portion of the signaling pathway.

Mouse Mammary Tumor Virus RNA is expressed in activated EL4.E1 lymphoma cells

Interest in the relationship between Mouse Mammary Tumor Virus (MMTV) and T cells in this laboratory was sparked by results obtained by a previous graduate student, Dr. John Elliot. He found that stimulation of the T lymphoma cell line EL4.E1 with PMA induced the expression of a novel 0.9 kb transcript corresponding to the 3' portion of the MMTV provirus (51). Accumulation of the transcript was inhibited by CsA. The pattern of induction and suppression of the novel MMTV mRNA was similar to that shown for IL2 and GM-CSF in these cells. Based on sequence analysis of cDNA clones and mRNA cap-labeling experiments, John Elliot determined that the PMA-induced transcript initiated from within a novel promoter in the envelope gene of endogenous MMTV (Figure 2-1) whereas constitutive expression of MMTV full-length and env mRNAs in these cells initiates from the conventional 5' LTR (Long Terminal Repeat) promoter/enhancer. Using the MMTV numbering of Moore et al (138), the PMA-induced transcript begins at or near position 7246. An intron spanning 1161 nt beginning 95 nt from the 5' end is spliced out. The remainder of the transcript is a copy of the 3' LTR which contains a 494 bp deletion characteristic of EL4.E1 provirus (Figure 1-2). Kwon and Weissman had earlier cloned a cDNA from PMA-stimulated EL4 cells which contained the same 3' sequence and LTR deletion but which included the 1161 nucleotide intron (115).

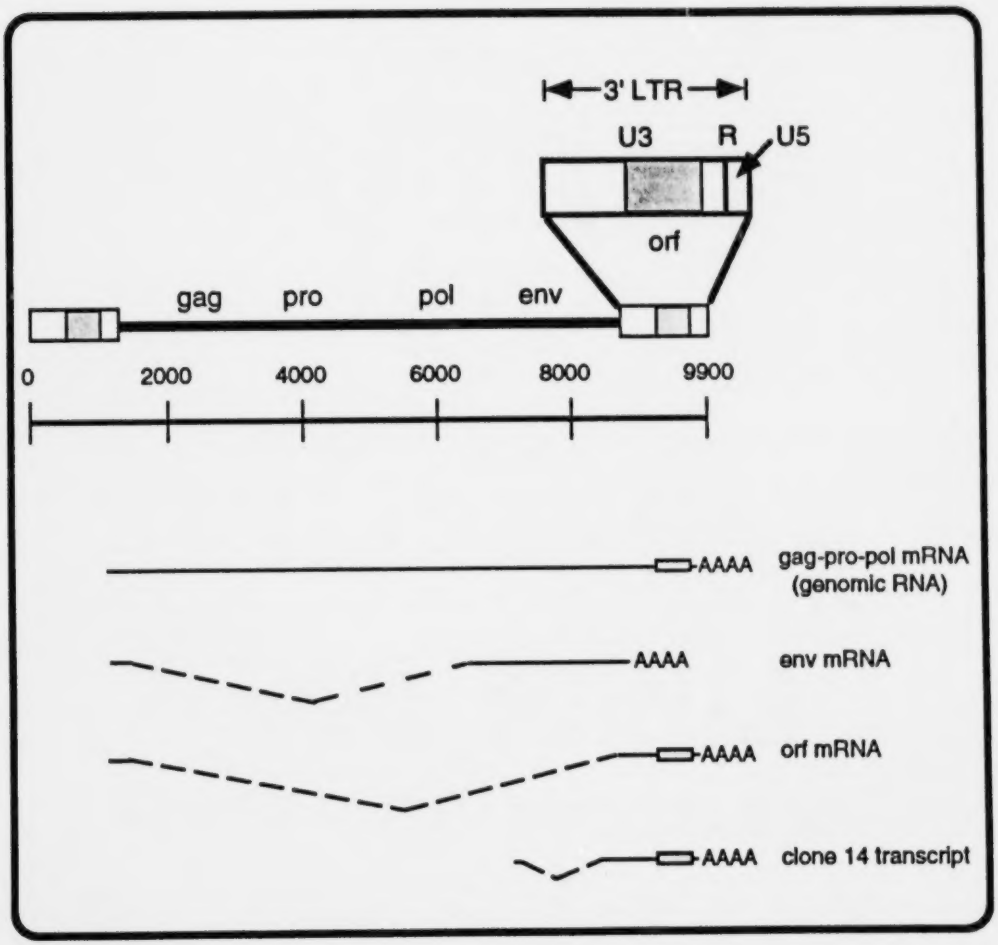
Our interest in MMTV arose from the shared transcriptional control with the IL2 gene, including CsA sensitivity. Furthermore the idea elaborated below, that MMTV may be involved in the generation of T lymphocyte tumors, warranted further investigation. The following sections describe some of the characteristics of MMTV including its role in the development of mammary tumors. Evidence supporting the hypothesis that MMTV is a causative agent of T lymphomas is also presented.

Discovery of MMTV

MMTV is a type B retrovirus which causes mammary adenocarcinomas in mice. Certain strains develop mammary tumors at a high frequency and hence were classed as high-incidence strains. It was demonstrated by Bittner in the mid-1930s that when newborn mice of a low-incidence strain were nursed by a foster mother of the high-incidence strain, most of the

Figure 1-2 General structure of MMTV provirus and RNA

The top diagram is a representation of the MMTV provirus showing the relative positions of the LTR and the coding regions for, gag, pro, pol, env, and 3' LTR orf (open reading frame). The expanded portion shows the 3' LTR with the U3, R, and U5 segments which are described in the text. The stippled box refers to the 494 bp deletion found in EL4.E1 cells. The lower portion are representations of the full-length RNA (genomic RNA and gag-pro-pol mRNA), the env mRNA, orf mRNA and the clone 14 transcript corresponding to the PMA-induced RNA described by Elliot et al (51). Areas spliced out of the mature RNAs are represented as dashed lines.


3' LTR
U3
R
U5
orf
gag
pro
pol
env
0
2000
4000
6000
8000
9900
AAAA
gag-pro-pol mRNA
(genomic RNA)
AAAA
env mRNA
AAAA
orf mRNA
AAAA
clone 14 transcript

female offspring developed mammary tumors. The infectious agent, later defined as MMTV, was known as the "milk-borne factor" (203). In addition to existing as an exogenous virion, MMTV is also present in one or more copies as endogenous provirus integrated at different chromosomal locations in virtually all strains of inbred mice. Most of the MMTV proviral loci (*Mtv*) are complete proviral units which are genetically stable and segregate independently (113, 119). While the majority of endogenous provirus are transcriptionally silent, some are conditionally expressed in certain tissues or in particular circumstances. In GR and DBA mice, *Mtv-2* has the capacity to produce infectious virus and to contribute to the development of mammary tumors (132).

Structure of the MMTV provirus

The general structures of the MMTV provirus and RNA are shown in Figures 1-2 and 1-3. The complete provirus is about 9900 bp in length including a 1300 bp LTR at each end. The viral genes encode the following proteins: gag-structural nucleocapsid proteins; pro-protease required for cleavage of viral polyproteins; pol-reverse transcriptase and integrase (IN); and env-envelope glycoproteins. The open reading frame (*orf*) in the U3 region of the 3' LTR, has recently been shown to contain the Mls (minor lymphocyte stimulating) locus which encode a class of minor histocompatibility antigens (2).

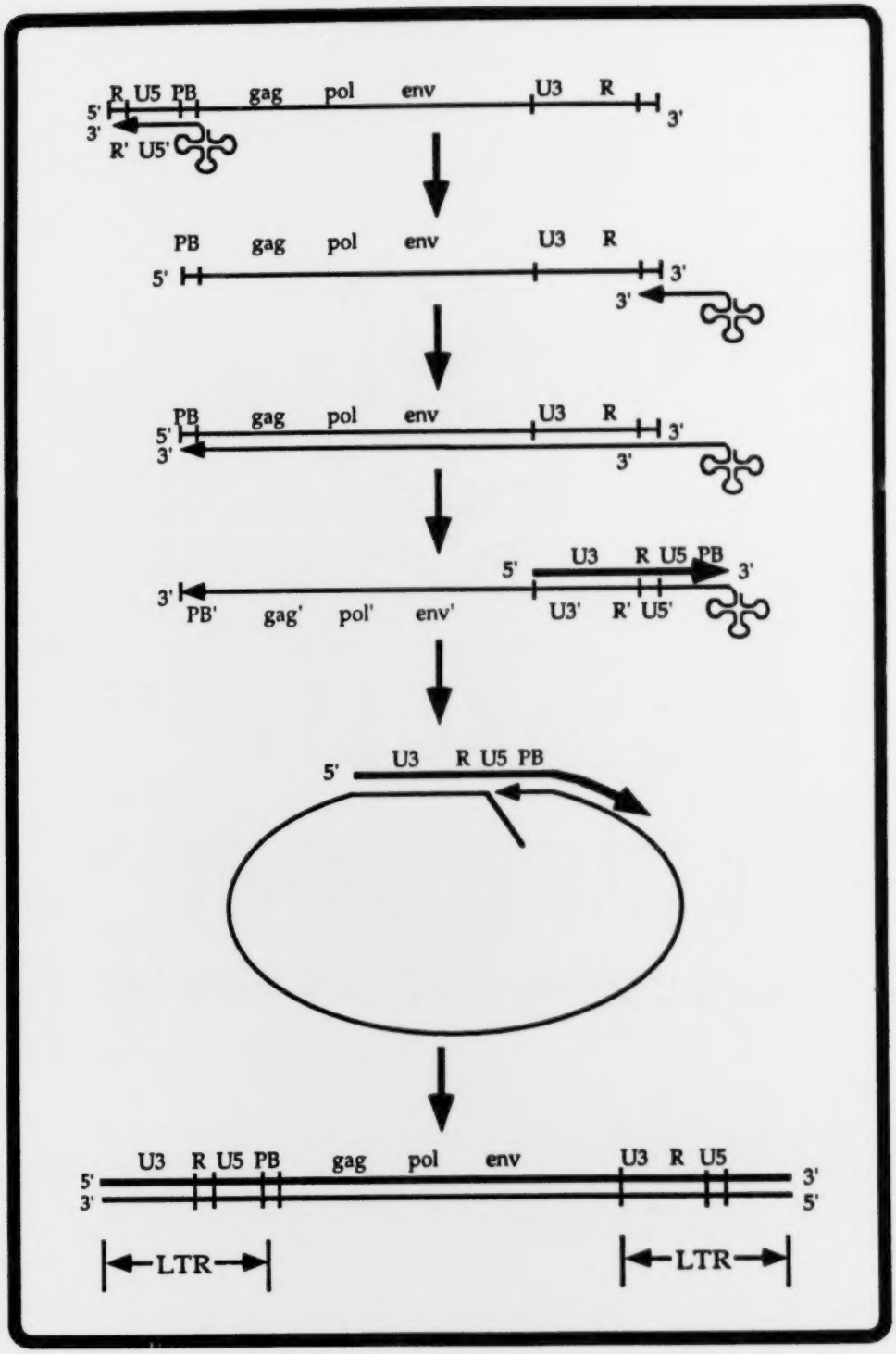
MMTV RNA has a terminally redundant 15 nucleotide segment (R) which is required to facilitate the transfer of nascent DNA from the 5' end of the viral genome to the 3' end. U5 and U3, individually unique sequences, contain both cis-acting elements required during replication and attachment sites necessary for integration of proviral DNA into the host cell chromosome. The primer binding site (PB) is base-paired with residues present in the 3' terminal region of a tRNA-lys primer. A leader region (not indicated) downstream from the PB site contains the splice donor site for env (envelope) and *orf* (open reading frame) mRNA, and a packaging signal.

Replicative Cycle of MMTV

The replicative cycle of infectious MMTV is similar to that followed by all retroviruses (32, 203). The first phase of retroviral replication occurs in the absence of viral gene expression, using proteins and enzymatic activities

Figure 1-3 Synthesis of retroviral DNA

The retroviral RNA genome is represented by a thin line. The negative DNA strand is indicated by a medium line and the positive DNA strand by a thick line. The cloverleaf structure represents a tRNA-lys primer and arrows indicate the direction of synthesis by reverse transcriptase. The relative positions of the gag, pol and env genes are indicated and the remainder of the terms are described in the text. The structure of the provirus is shown and the long terminal repeats (LTRs) are indicated. This figure is adapted from a diagram given in reference 32.


R U5 PB gag pol env U3 R
5'
3'
R' U5'
PB gag pol env U3 R
5'
3'
3'
PB gag pol env U3 R
5'
3'
3'
3'
PB' gag' pol' env'
5'
U3 R U5 PB
3'
U3' R' U5'
5'
U3 R U5 PB
U3 R U5 PB gag pol env U3 R U5
5'
3'
3'
5'
LTR
LTR

associated with the virion at the time of infection. The extracellular virus contains two RNA positive-strand genomes linked together at their 5' ends with a molecule of virally-encoded nucleic acid binding protein. The RNA genome, like eukaryotic cellular mRNA, is capped at the 5' end and polyadenylated. The genomes are associated with a core of viral protein which in turn is surrounded by an envelope derived from host cell plasma membrane.

Infection is mediated by the interaction of viral glycoproteins, present on the envelope surface, with specific receptors on a permissive cell and internalization likely occurs by receptor-mediated endocytosis. Within the cytoplasm, the RNA is transcribed into a DNA intermediate using reverse transcriptase associated with the nucleoprotein complex. The general mechanism of proviral DNA synthesis is shown in Figure 1-3. Within the activated nucleoprotein complex, formation of the DNA replication intermediate is initiated by synthesis of a negative DNA strand by reverse transcriptase. This synthesis is primed by a tRNA-lys molecule which is base paired at its 3' end to the PB site bordering the 3' end of U5. Synthesis continues through U5 and the 5' R region. The nascent DNA strand, presumably still associated with reverse transcriptase, is transferred to the 3' end of the genome and by base-pairing to the R sequence. This allows synthesis of the negative DNA strand to continue towards the 5' terminus of viral RNA template. The RNA template is removed by the RNase H activity of reverse transcriptase.

Priming on the newly synthesized negative DNA strand is required for formation of the positive strand. To generate a primer, reverse transcriptase cleaves the RNA strand, present in the DNA-RNA hybrid, within a polypurine tract located just 5' of the U3 region of the RNA. Once copying of the positive strand to the U5' terminus is complete, another 'jump' or transfer is required. Correct positioning of the positive strand on the negative strand template occurs by base pairing between complementary PB site sequences. The tRNA primer and remnants of the viral RNA genome are removed, likely by RNase H activity of reverse transcriptase. Completion of both the positive and negative DNA strands yields the provirus in which the gag, pol, and env coding regions are flanked by LTRs.

The proviral DNA, associated with proteins including virally-encoded integrase (IN), is transported into the nucleus prior to integration

within the host chromosome. Integration, requisite for the completion of the replicative cycle, results in shortening of the provirus by loss of 2 bp at each LTR terminus and duplication at the site of insertion of 4-6 bp of cellular DNA. In vitro experiments with integrase of Moloney murine leukemia virus (36) and avian sarcoma-leukosis virus (104) suggest that this enzyme has all the enzymatic properties required for correct cleavage of the attachment sites on the proviral DNA, cutting of host chromosomal DNA, and ligation of the viral DNA to the target DNA. Although the site of retroviral integration is considered to be more or less random, evidence suggests that insertion may occur at preferred sites possibly those which are transcriptionally active or which correspond to a relatively open chromatin structure. Chromosomal position effects are important for the transcription potential of newly-integrated MMTV (56, 198). Once integrated, the provirus is considered to be genetically stable and no known mechanism exists for its subsequent excision and transfer to another chromosomal location (32).

Completion of the infectious cycle requires expression of the MMTV provirus using transcriptional and translational enzymes and substrates provided by the host cell. Viral RNA, transcribed by host RNA polymerase II using as template the provirus integrated into the host cell genome, is capped at the 5' end and polyadenylated analogous to cellular mRNA. It is not known how full-length viral RNA is segregated and committed for use either as genomic RNA or as gag-pro-pol mRNA for translation. Processing and modification of viral proteins is followed by assembly of the nucleoprotein core structure, association of it with portions of the plasma membrane enriched in viral glycoproteins, and budding from the cell surface. The immature nucleoprotein core structure of MMTV, when present intracellularly, are called A-type particles. Infection with MMTV is not generally immediately detrimental to the cell.

Expression of MMTV RNA

Efficient expression of MMTV provirus is dependent on interaction of eukaryotic transcriptional factors with cis-acting elements contained within the LTR. Conventional transcription is initiated in the 5' LTR near the junction of U3 and R (about 135 nucleotides upstream of the LTR-gag junction). The LTR contains binding sites for the transcription factors nuclear factor-1 (NF-1) and a TATA binding protein, likely TFIID (34, 194).

The MMTV promoter is under the transcriptional control of a set of enhancer sequences, termed the glucocorticoid response elements (GRE), that mediate positive transcriptional regulation by the binding of hormone-receptor complexes (reviewed in 12). The GRE has been localized to a region which spans approximately 200 nucleotides upstream of the major LTR transcriptional start site (-202 to -59) and is composed of multiple binding sites for the hormone activated receptor (22, 24). Hormone-receptor association with the GRE results in an alteration of chromatin structure allowing accessibility of NF-1 and other transcription factors to their respective binding sites (4, 152).

MMTV transcription is modulated by additional positive and negative regulatory elements (NRE) which may act synergistically with the GRE to effect cell-specific expression. The highest level of MMTV expression occurs in the epithelial cells of the mammary glands of female mice during pregnancy and after parturition. Significantly lower levels of transcription were detected in salivary glands, kidneys, lungs, seminal vesicles and lymphoid cells of the spleen and thymus (86). In vitro studies suggest that NRE are present in the region spanning -560 to -631 (136) and repressive activity has also been associated with sequences lying between -364 and -438 (136, 121). Regions upstream of these elements may be important for positive regulation of LTR transcription (121, 136). Ross and coworkers examined expression from the MMTV LTR in transgenic animal using full-length or deletion-containing LTRs linked to reporter genes (166). The hybrid transgenes containing the full-length LTR were expressed in mammary tissue, salivary glands, thymus, lung and spleen, consistent with the tissue-restricted pattern of expression shown for endogenous MMTV (86). Deletion of the region upstream of -364, or deletion of sequences between either -201 and -471 or -201 and -344 resulted in expression of reporter genes at novel tissue sites such as brain, heart and skeletal muscle in animals expressing the various transgenes (166). Taken together these results support the conclusion that the MMTV LTR contains one or more NREs which prevent inappropriate expression of the provirus in different tissues.

MMTV is a causative agent of mammary adenocarcinomas

Development of mammary adenocarcinomas in high-incidence mouse strains is associated with chronic infection by MMTV. Infected C3H

mothers express high titres of MMTV in their milk and about 90% of the female offspring develop mammary tumors between seven and ten months of age. If C3H offspring do not receive exogenous virus, which is attained by fostering on virus-free strains, mammary tumors develop in only 20%-40% of female mice between 18 and 24 months (137). Tumor development requires multiple pregnancies and is linked to endogenous MMTV production from a locus called *Mtv-1*. In GR mice, expression of MMTV in mammary tissue is associated with expression of *Mtv-2* locus and is directly implicated in the development of mammary tumors (132). Tumorigenesis associated with *Mtv-1* and *Mtv-2* occurs as a result of reintegration of MMTV provirus generated from these vertically transmitted MMTV provirus.

Activation of cellular proto-oncogenes by MMTV promoter/enhancer

The MMTV genome does not contain a known oncogene and appears to act as a retroviral insertional mutagen. Mammary tumors which arise clonally in susceptible mouse strains frequently have one or more MMTV provirus, in addition to endogenous copies unique to that particular mouse strain, integrated within specific regions designated int loci. Developmentally inappropriate, or augmented expression of the int proto-oncogenes is thought to occur as a result of activation of the MMTV LTR promoter/enhancer.

Analysis of mammary tumors from high incidence strains C3H and BR6 led to the identification of two cellular loci designated *int-1* (now renamed *Wnt-1*) and *int-2*, which are often flanked or occupied by MMTV provirus (41, 147, 150). The oncogenic potential of the *Wnt-1* and *int-2* has been established by several lines of work. Transfection of non-tumorigenic mammary cell lines with plasmids containing genomic *Wnt-1* transcriptionally activated by several different retroviral promoters led to altered cell growth (21) and tumorogenicity in syngeneic mice (163). Tsukamoto and coworkers constructed a gene in which the MMTV proviral LTR was placed in the opposite transcriptional orientation 5' to a genomic *Wnt-1*. Expression of the transgene allele was associated with the development of mammary and salivary adenocarcinomas in male and female mice (197). Similarly, expression of the int-2 gene driven by the MMTV LTR promoter/enhancer resulted in mammary gland hyperplasia in transgenic

females (142). *Wnt-1* and *int-2* may act cooperatively in the genesis of mammary carcinomas. Transgenic mice carrying either the *Wnt-1* or *int-2* transgene under the control of the MMTV LTR were bred to obtain doubly transgenic animals. Mammary tumors arose earlier and with a higher frequency in both male and female bitransgenic animals than in littermates bearing only a single transgene (114).

Most mammary tumors do not contain both mutated *Wnt-1* and *int-2* loci and a significant number do not express either mRNA, suggesting that other loci may be relevant in MMTV induced carcinogenesis (53, 151, 165). An additional locus, *int-3* was shown to be disrupted by MMTV proviral insertion in mammary tumors isolated from the Czech II mouse strain (69, 68). Transgenic animals expressing the int-3 gene linked to the 3' region of MMTV (containing the *env* gene and the 3' LTR) develop hyperplasia in the epithelia of mammary and salivary glands and focal adenocarcinoma in these tissues (99).

Gene products encoded by *Int* genes

Mapping of MMTV provirus in tumors expressing one or more int loci shows that insertions often cluster within 5 kb upstream or downstream of the respective *int* coding region (68, 151, 202). Provirus located 5' of each gene are usually in the opposite transcriptional orientation, while downstream insertions are in the same orientation as the gene. Although provirus can integrate into introns and untranslated sequences the protein-coding domains of the int genes remains intact (151). Newly-acquired MMTV proviral copies in mammary tumors appear to function as enhancers which increase int gene expression as opposed to acting as a promoter insertional mutagens. Mapping of RNA start sites indicated that in the majority of mammary tumors studied, transcription was initiating correctly from within the *Wnt-1*(146) or *int-2* (40) promoter.

The *int* genes characterized to date are present on different chromosomes and encode proteins with little or no homology to one another. The *Wnt -1* gene is normally not expressed in mammary tissue but instead expression is limited to adult mouse testes and the neural tube of mid-gestational embryos (175). A role for the *Wnt-1* protein in CNS development is supported by the finding that in embryos homozygous for a mutant *Wnt-1* allele most of the mid-brain and cerebellum was missing (128). The

Drosophila homolog of *Wnt-1* is encoded by the wingless gene. The normal functioning of this gene is required for correct pattern formation within each segment in the developing fly embryo (162). *Wnt-1* encodes a glycoprotein which may be involved in intracellular signaling during CNS development (100). The *int-2* gene encodes a 27 kDa protein with homology to members of the basic fibroblast growth factor family and is a mitogenic signal in the development of the inner ear (161). The *int-3* gene appears to encode a transmembrane protein with homology to the neurogenic *Notch* gene of *Drosophila* (99). These observations indicate that *Wnt -1*, int-2 and int-3 gene expression is morphogenic during normal mouse embryonic development. Inappropriate expression of these gene products may initiate a multi-step oncogenic process by altering the proliferative potential of mammary epithelial cells.

The *orf* gene of MMTV corresponds to the Mls loci

The Mls (minor lymphocyte stimulating) antigens were originally identified by their ability to stimulate proliferation in mixed lymphocyte cultures of splenic cells taken from mouse strains of different genetic backgrounds, but matched at the MHC (57). Along with certain bacterial exotoxins which are also potent T cell mitogens, the Mls determinants have been termed superantigens (reviewed in 88). Conventionally, T helper cells are stimulated as a result of specific recognition by the TcR of peptide antigens bound to the antigen-binding groove of MHC Class II complex on an APC. In contrast, although interaction with MHC Class II on the APC is required the Mls protein stimulates T cells, by the recognition of certain classes of Vβ chains present in the TcR. They have the potential to activate T cells that express one or more of the 20 or so genes encoding the TcR β chain. In cell culture, this interaction results in T lymphocyte activation and proliferation. Expression in vivo results in the deletion of thymocytes bearing reactive Vβ elements within the thymus during the establishment of tolerance. It is thought that B cells are important as APC (73), but T cells also present the Mls superantigen (117, 207). $CD8^+$ T cells are more effective than $CD4^+$ T cells, although either are competent in vivo.

The Mls loci were characterized before the potential gene products were identified. It was observed that Mls-like activity mapped close to, or at the same chromosomal locations as several Mtv loci (48, 61, 211). For exam-

ple, Mls-1 cosegregates with *Mtv-7* and has specificity for T cells bearing Vβ6, Vβ8.1 and Vβ9 (61). It was recently shown that infectious milk-borne virus of C3H/HeJ codes for a superantigen that interacts with T cells bearing Vβ14 and Vβ15. Further experimentation provided definitive proof that the *orf* gene within the 3' LTR encodes a product that directly or indirectly confers the ability of the expressing cell to interact with specific Vβ elements. Transfection experiments have shown that expression of the *orf* gene in a suitable APC results in the activation and IL2 secretion from cocultured T hybridomas cells lines bearing the appropriate Vβ TcR (212, 153, 28). Acha-Orbea et al. have shown, using a transgenic model, that the ORF protein encoded by *Mtv-2* of GR mice causes the deletion of Vβ14$^+$ T cells (2). The kinetics of clonal deletion were slow, and complete clonal deletion was not observed until the transgenic animals reached 4-5 months of age. Transgenic mice expressing the ORF glycoprotein derived from C3H exogenous MMTV also efficiently deleted Vβ14$^+$ thymocytes (74). Clonal deletion occurred earliest in mice expressing higher levels of the transgene. It is evident that the Mls determinants encoded by either germline *Mtv* loci or by exogenous milk-borne virus are not equivalent in their ability to elicit a T cell response. Although *Mtv-9*, encoding the Etc-1 superantigen, causes the deletion of Vβ5+ and Vβ11+ cells in vivo, it has not been possible to generate an MLR response with cells carrying it (73). It is likely that the kinetics and magnitude of the T cell response to the Mls determinants is dependent on a number of factors including its level of expression, structurally differences between the various ORF superantigens and the genetic and immunological characteristics of the host.

The levels of *orf* mRNA detected in certain tissues is very low, and the ORF protein has not been detected in infected tissue or in primary tumor cells. Racevskis used antisera raised against a synthetic MMTV ORF peptide to immunoprecipitate several glycoproteins from PMA-treated EL4.E1 lymphoma cells and demonstrated that the non-glycosylated protein was 21 kDa in size (154). It is possible that the PMA-induced transcripts identified in EL4 cells are translated into a truncated Mls-related protein (51, 115).

Translation of MMTV RNA containing the *orf* gene in vitro generates a predominant protein 36 kDa in size which is glycoslylated to yield a 46 kD glycoprotein (19, 112). Translational studies using canine pancreatic microsomes indicate that the Mls product is an integral membrane protein

with a short, N-terminal segment (30-40 amino acids) and with the C-terminus forming an extracellular domain (27, 112). A series of experiments using *orf* genes truncated in the N terminus showed that at least 38 amino acids could be deleted without significantly impairing the ability of the protein to elicit a T cell mitogenic response in vitro (27). Specificity of interaction with the Vβ elements appears to lie within the C-terminal domain of Mls protein. Sequence analysis of *orf* genes from *Mtv* loci associated with the deletion of T-cells bearing the same subset of Vβ show that they are nearly identical in sequence, whereas in *Mtv* loci with different specificity there is divergence amongst 30 amino acids at the C-terminus (28, 112, 153).

It is unclear whether production of superantigens from endogenous provirus present in the germline are of any benefit to the mouse. Deletion of Mls-responsive thymocytes during thymic selection could potentially result in gaps in the T cell repertoire. It is possible that the endogenous provirus are simply remnants of infective viruses which have accumulated in the germline over time.

Lymphocytes may be carriers of MMTV

There is evidence that lymphocytes are involved in the transport of milk-borne MMTV from the intestine to mammary glands. Neonatal thymectomy decreases the incidence of mammary tumors in mice receiving exogenous MMTV virus (184). Tsubura and coworkers found that injection into nude mice of a T cell enriched population derived from an animal infected with the milk-borne virus resulted in MMTV protein expression in mammary epithelium (196). Injection of natural killer cells, B cells, or macrophages was ineffective. In transgenic animals, high levels of expression of the C3H ORF protein were correlated with efficient deletion of $V\beta14^+$ T cells. These animals were resistant to infection with the C3H milk-borne virus (74), suggesting that T cells can serve as carriers of MMTV. The mechanism of T cell involvement has been postulated to be as follows. Immune cells become infected with MMTV near the site of viral entry in the gut (1). Infected B-cells, expressing MHC Class II and an Mls determinant, may stimulate nearby T cells bearing the appropriate Vβ elements leading to their activation and proliferation. T cell activation may then facilitate integration of MMTV provirus, and amplify the number of lymphocytes harboring the provirus. Therefore lymphocytes may serve as a reservoir of MMTV,

with the capacity to produce virus for infection of mammary tissue during lactation.

Relationship of MMTV to the development of T cell lymphomas

In addition to its role in mammary tumor formation MMTV is also associated with the induction of T cell lymphomas. Male GR mice receiving exogenous virus transmitted through nursing develop thymic leukemias, albeit at a slower rate and with a lower frequency than female littermates develop mammary tumors (134). Lymphomas develop around 13 months of age at an incidence of about 20%. The incidence is lowered to about 8% in male mice which are foster-nursed on non-viremic mothers. It is hypothesized that leukemic development is associated with expression from the *Mtv*-2 locus, supported by the finding that in congenic GR mice lacking *Mtv*-2 development of lymphomas is rare.

Thymic leukemic cells derived from the GR strain, although they do not appear to shed infectious virus, are characterized by high MMTV RNA expression, production of precursor proteins and additional newly-acquired copies of MMTV provirus (133, 134). These amplified MMTV integrants are present in the primary leukemia cells and behave as stable elements during serial transplantation in vivo or during culture in vitro (133, 134). Based on restriction enzyme analysis, these amplified provirus appear to be derived by expression from the *Mtv*-2 locus.

Amplification of MMTV proviral DNA has also been reported in T leukemias of DBA/2 (120,214), BALB/c (93) and C57BL/6 (51, 93) mouse strains. In general the lymphomas contain more new MMTV integrants than MMTV-induced mammary tumors. A striking feature characteristic of these and of GR leukemias, is the presence of deletions within the U3 region of the LTR within the newly-acquired MMTV proviral DNA. The structural alterations consist either of a 350 bp to 450 bp deletion, or a deletion and substitution of additional sequence. Within each tumor, the deletion is identical and therefore appears to have arisen as a result of a mutational event occurring prior to the amplification of the provirus within the host cell. Deletions within the LTR, although characteristic, are not unique to MMTV-expressing T cell lymphomas, and have been found in kidney carcinomas, pancreatic tumors and other non-mammary cells (156, 209). A sam-

pling of the reported deletion-containing LTRs is shown diagramatically in Figure 1-4.

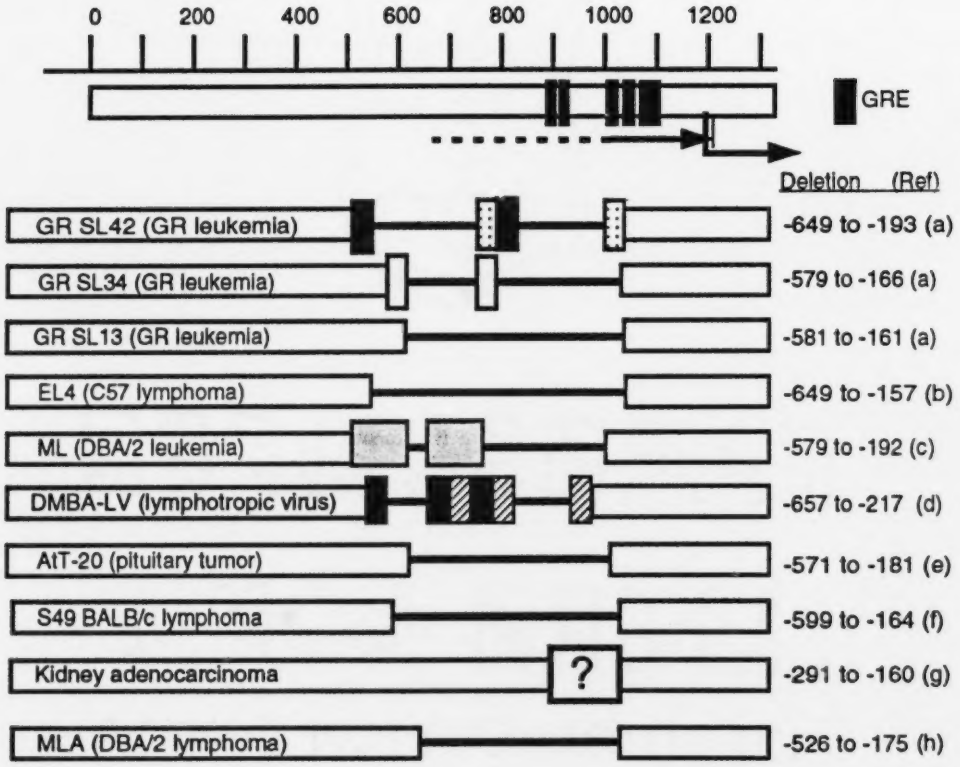
The altered MMTV LTR present in the kidney adenocarcinoma (listed in Figure 1-4) is unique in that the size of the deletion is comparatively small; 113 bp have been removed and replaced with 91 bp of unknown sequence (209). Kidney tumors develop with an incidence of 70% at 19 to 25 months of age in the mouse substrain BALB/cfC3H. These mice were selected from a colony in which BALB/c mice were fostered nursed on C3H mothers transmitting C3H exogenous MMTV in their milk. Tumor cells produce MMTV proteins and viral particles indistinguishable from exogenous C3H MMTV. The authors speculated that changes within the LTR have resulted in a new virus with altered target cell specificity.

It is believed that deletions within the LTR of amplified MMTV provirus may remove or alter important transcriptional control elements and contribute to T cell tropic expression of MMTV and potentially to the development of MMTV-induced neoplasia. Alterations in cell-specific expression could result from a loss of one or more NREs or by the creation of positively-acting enhancer elements. These mechanisms are not mutually exclusive and there is evidence suggesting that both may be relevant (120, 133, 135, 188, 214). Deletion-containing LTRs derived from EL4 cells and from a BALB/c lymphoma (Figure 1-3), when tested in a transient transfection assay in mink lung cells, showed a higher level of basal activity in the absence of glucocorticoid hormone stimulation than shown by the full-length LTR (93). These results suggest that in certain T lymphomas, deletions within the LTR may remove regulatory elements involved in tissue-specific expression.

Additional studies have been done in which deletion-containing LTRs have been placed upstream of the CAT reporter gene. These constructs were introduced into both murine and human T cell lines. Theunissen and coworkers demonstrated that two deletion-containing LTRs isolated from GR leukemic cells were active in several T cell lines which had been treated with PMA. Although they were unable to detect enhancer-like activity in any MMTV fragments isolated from the altered LTRs, it was suggested that the LTR rearrangements resulted in the generation of a PMA-responsive element which could contribute to T cell specific expression of MMTV (188). Another group has suggested that LTR deletions occurring in two DBA/2

Figure 1-4 Positioning of deletions within the MMTV LTR of tumor cells and a thymotropic virus

The MMTV LTR, approximately 1300 bp in size is shown in the top panel. The GRE elements (black boxes) and the LTR transcriptional start site at 1195 are indicated. The LTR deletions are represented as a solid line, and boxes within the deletion represent flanking sequences which are duplicated and inserted into the resulting mutated LTR. The size and position of the deletion is given with respect to the 5' LTR promoter start site. Additional detail can be obtained from the cited references. The LTR deletions were described in the following references, a- ref. 133; b-ref 51; c- ref. 120; d- ref. 9; e- ref 156; f- ref 93; g- ref 209; h- ref. 214.


0
200
400
600
800
1000
1200
GRE
Deletion (Ref)
GR SL42 (GR leukemia)
-649 to -193 (a)
GR SL34 (GR leukemia)
-579 to -166 (a)
GR SL13 (GR leukemia)
-581 to -161 (a)
EL4 (C57 lymphoma)
-649 to -157 (b)
ML (DBA/2 leukemia)
-579 to -192 (c)
DMBA-LV (lymphotropic virus)
-657 to -217 (d)
AtT-20 (pituitary tumor)
-571 to -181 (e)
S49 BALB/c lymphoma
-599 to -164 (f)
Kidney adenocarcinoma
?
-291 to -160 (g)
MLA (DBA/2 lymphoma)
-526 to -175 (h)

lymphoma cell lines resulted in both the loss of an NRE and the emergence of an enhancer-like sequence (214). Both of the altered MMTV LTR sequences tested were active in T cell lines and expression was moderately enhanced by dexamethasone treatment. The LTR isolated from the DL-8 cell line was also active in a human mammary cell line in the absence of hormone. Deletion analysis suggested an enhancer element was present within the region spanning 525 to 558 (-670 to -637). This segment stimulated transcription from either the MMTV promoter or a heterologous promoter.

The deletions found in various MMTV isolates studied remove or alter sequences present between approximately positions -150 and -650 with respect to the LTR transcriptional start site (Figure 1-4). Additional mutational analyses, discussed in the section "Expression of MMTV RNA", indicate that one or more NRE are present within this segment and function in different tissues to inhibit expression of MMTV RNA. Therefore, it is likely that a specific LTR-deletion within amplified provirus in a T lymphoma removes or alters NRE which then facilitates expression of MMTV within these cells. In the majority of cases it is difficult to determine whether alterations within the LTR result in the creation, or de-inhibition, of an existing positive enhancer element which are functional in T lymphocytes. A deletion-containing LTR derived from EL4 cells was active in transgenic animals at the same tissue sites as the full-length LTR. Although it was functional in additional tissues, suggesting the loss of an NRE, there was no evidence of enhanced expression in T lymphocytes.

The above evidence links MMTV to T lymphomagenesis only circumstantially. Strong, direct evidence implicating MMTV involvement in the development of T lymphomas is provided by the characterization of an MMTV-related thymotropic virus, DMBA-LV (8, 9). This transmissible virus induces T cell thymomas with a high incidence and a short latency period. In one study, 90% of the animals had thymic tumors 42 days post-injection and infection was abolished by a monoclonal antibody directed against an MMTV glycoprotein (8). Leukemic cells have newly-acquired provirus which contain a characteristic deletion within the LTR consisting of a 440 bp deletion and a 122 bp insertion comprised of sequences which flank the substituted region (Figure 1-4).

Thesis objectives and hypothesis

As outlined in this introduction, there is a strong association between MMTV and cells of the immune system. Lymphoid cells present and respond to the Mls superantigen encoded by the MMTV LTR-ORF. Furthermore, MMTV has been implicated in the development of T lymphomas. It was previously determined by this laboratory that a novel promoter is present in the *env* gene of amplified MMTV provirus in EL4.E1 cells (51). Transcription from this promoter is induced by the same signals which result in IL2 production, and accumulation of both transcripts is blocked by CsA. We hypothesize that the MMTV *env* promoter is involved in the regulated expression of MMTV and may contribute to MMTV-induced lymphomagenesis.

The objectives of the thesis were to:

1. isolate the MMTV *env* gene segment containing the novel promoter/enhancer from EL4.E1 cells, and to link this segment to a CAT reporter gene to be used in transient transfection assays.
2. determine whether the MMTV *env* transcriptional activator (META) segment was functional only in helper T cells, or whether it could also be expressed in other types of cells.
3. determine if META activity in T helper cells was induction-dependent and CsA-sensitive.
4. identify potential transcription regulatory sequences important for the inducible, CsA-sensitive, tissue-specific expression of META.

CHAPTER TWO. MATERIALS AND METHODS

Reagents

Molecular biological reagents and enzymes were obtained from Gibco BRL; Life Technologies, Burlington, Ontario. Restriction enzymes were used in buffers provided by the manufacturer. Phorbol 12-myristate 13-acetate (PMA), Concanavalin (Con A) and ionomycin were obtained from Sigma Chemical Co. (St. Louis, MO.) and stock solutions of PMA and ionomycin were made up in dimethylsulfoxide (DMSO). Cyclosporin A (CsA) was a gift from Sandoz Canada Inc., Dorval, Quebec. Radionucleotides, [γ-^{32}P] adenosine 5'-triphoshate tetra-(triethylammonium) salt, [α-^{32}P] deoxycytidine 5'-triphoshate tetra-(triethylammonium) salt and [Dichloroacetyl-1,2-^{14}C] Chloroamphenicol were obtained from New England Nuclear, Lachine, Quebec.

Cell Lines and Culture Conditions

Cell lines were maintained in RHFM consisting of RPMI 1640 (Gibco BRL) supplemented with 20 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid [HEPES; pH 7.4], 100 µM 2-mercaptoethanol, 100 µg/ml streptomycin, 100 IU/ml penicillin and 10% fetal bovine serum (FBS; Hyclone). Mouse cytotoxic T cell lines (CTL) were maintained in RHFM containing 30 U/ml human recombinant IL2 (11). Cell lines used in the study were: S194 (mouse myeloma), P815 (mouse mastocytoma), EL4.E1 (C57Bl/6 mouse T lymphoma, ref 54), MTL2.8.2 (mouse CTL, ref. 16), Jurkat (human T leukemia), LBB 3.4.16 (B cell hybridoma, ref. 144) and 12.1.19 (T-cell hybridoma, ref. 59). Cells were grown in 75 cm^2 tissue culture flasks (Corning, Corning N.Y.) at 37 ^{o}C with 5% CO_2 in a humidified incubator.

Several T helper cell lines were used in the study. EL4.E1 cells secrete IL2 in response to stimulation by PMA (15 ng/ml) alone or IL2 production can be enhanced by costimulation with ionomycin or the calcium ionophore, A23187. The human T cell line, Jurkat requires two stimuli to maximally induce IL2 production; PMA (10 ng/ml) was used in combination with either Con A (10 µg/ml) or ionomycin (1.5 µM). The T cell hybridoma 12.1.19 was activated by incubation with antigen (poly 18) and suitable antigen presenting cells, with anti-CD3ε (59) or by inclusion of Con A. For example, in a typical experiment using Jurkat cells, a stock solution contain-

ing 900 µg of Con A, 300 ng of PMA and 3 µg of CsA in a 5 ml volume was added to 25 mls of transfected cells in culture to achieve a final concentration of 30 µg/ml Con A, 10 ng/ml PMA, and 100 ng/ml CsA.

Transfection procedure

Transfections were done using a modified DEAE-dextran procedure (63). All plasmid DNA used in the study was greater than 90% closed circular DNA as assayed by ethidium bromide fluorimetry (139). Cells in log growth phase were washed twice in serum-free medium and resuspended in Tris-Buffered Saline (TBS: 25 mM Tris HCl, pH 7.5, 137 mM NaCl, 5 mM KCl, 0.6 mM Na_2HPO_4, 0.7 mM $CaCl_2$ and 0.5 mM $MgCl_2$). Fifteen µg of plasmid DNA was diluted in 0.5 ml TBS and then 0.5 ml of a 2000 µg/ml stock solution of DEAE-dextran (Diethylaminoethyl-Dextran, m.w. 500,000, Sigma) was added. Dilution of the concentrated plasmid preparation in TBS prior to the addition of DEAE-dextran prevented precipitation of the DNA. $1.5x10^7$ cells in TBS were then slowly added to achieve a final volume of 2 ml. The relative proportions of plasmid/cells/total volume was equivalent in all experiments. After incubation for 30 min at room temperature, the cells were washed once in RHFM and recultured at $2x10^5$ per ml.

Following transfection, cultures were incubated for up to 45 hr prior to harvest. Stimulating (PMA, ionomycin, other mitogens) or other (CsA) agents were usually added for the final 12 to 15 hr of culture. Cells were transfected using recombinant plasmids containing the reporter gene, Chloramphenicol acetyltransferase (CAT). Unless otherwise indicated, following the transfection procedure all cells were harvested and cellular extracts prepared for use in CAT assay. Specific experimental conditions are presented with each experiment.

The numbers of viable cells recovered at the conclusion of each transient-expression experiment varied. Factors affecting viability included: (1) Variable toxicity of DEAE-dextran and the transfection protocol on the different cell types tested. (2) Toxicity (non-specific or otherwise) associated with the use of mitogenic agents such as Con A and PMA. (3) Induction of programmed cell death (apoptosis) in response to stimuli required for IL2 production. For example when the T-hybridoma cell line, 12.1.19 is activated by antigen, by Con A or by anti-CD3ε significant cell death occurs within 12 hr post-activation.

Chloramphenicol Acetyltransferase Assay

At harvest, cells were washed once in cold phosphate buffered saline (PBS) and transferred to a microcentrifuge tube with 1.5 mls of PBS. The tubes were spun for 30 seconds at 14000 rpm in a refrigerated Eppendorf microfuge (Brinkman) and the pellet was resuspended in 0.1 ml of cold 0.25 M Tris-HCl, pH 7.5. The samples were placed on dry ice for at least 5 min and then thawed for 5 min at 37°C and vortexed well. After three freeze-thaw cycles, the preparation was spun for 5 min at 14,000 rpm at 4°C and the supernatant was stored at -70°C. Protein concentrations were determined using Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, Richmond, CA). Serial dilutions of bovine serum albumin (BSA) (Sigma, Fraction V) ranging in concentration from 100 µg/ml to 1.56 µg/ml were used as standards.

The CAT assay was performed essentially as described by Gorman (76). The assay involves incubation of cellular extracts with radioactive Chloramphenicol and acetyl coenzyme A (acetyl CoA), separation of acetylated Chloramphenicol products by thin layer chromatography (TLC) and quantitation by liquid scintillation counting. The proportion of acetylated products formed was taken to reflect the level of expression from the transfected plasmid.

An equivalent amount of protein was used for every sample within each experiment. Up to 70 µL of cell extract was incubated with 0.02 µCi [^{14}C] Chloramphenicol, 0.53 mM acetyl CoA (Pharmacia, Uppsala, Sweden), and 0.25 M Tris-HCl, pH 7.5, in a total of 150 µL for 3 to 4 hr at 37°C. Acetylated products were extracted by the addition of 1 ml ethyl acetate and the phases were separated by centrifugation at 14,000 rpm for 5 min at room temperature. The organic solvent layer was transferred to another microcentrifuge tube and dried down in a SpeedVac (Savant). The samples, resuspended in 30 µl of ethyl acetate, were spotted onto a TLC plate (silica gel precoated plastic sheets 60 F_{254}; E. Merck, Darmstadt, Germany). The chromatography tank containing 150 mls of 19:1 chloroform:methanol solvent was equilibrated for 2 hr prior to chromatography. The plates were placed into the tank for approximately 15 min or until the solvent front reached the top of the TLC plate. The plates were air-dried and the results were visualized by autoradiography using Kodak X-OMAT AR film (Eastman Kodak, Rochester, NY).

There were up to five spots for each sample. They were, in ascending order, a weak spot at the origin, non-acetylated Chloramphenicol, two monoacetylated forms and possibly a diacetylated product. To determine the amount of radioactivity in each spot, the TLC plate was overlaid with transparent tape, and using a light box and the autoradiograph for reference, the TLC sheet was cut. Individual pieces were placed in a scintillation vial with 0.5 ml of methanol. After ensuring the silica coating was saturated with methanol, 8 ml of aqueous counting scintillant (Amersham, Oakville, Ontario.) was added and quantitation was done in a Beckman Scintillation counter. The activity was calculated as follows: percent acetylated = counts in acetylated species/ counts in acetylated species + counts in nonacetylated chloroamphenicol.

Quantitation of CAT enzymatic activity

Recombinant plasmids containing the CAT coding region linked to DNA sequences of interest have been extensively used in transient-expression assays to study basal promoters and both positive and/or negative regulatory elements. Various methods have been used to normalize CAT enzymatic activity and allow comparisons of the relative strengths of sequences being tested. Comparisons can be based on protein content, cell number (total or viable), or the activity of another enzyme. In experiments such as these the protein content may not be proportional to cell number due to variations in the size following stimulation. Also viability may change as stimulation can induce cell death by apoptosis. Thus, the relative increase in CAT activity may vary depending upon the basis of comparison. It is beyond the scope of this thesis to discuss the relative merit of each of the methods. In this thesis I have chosen to use an equivalent amount of protein for each sample within a given experiment and to present the CAT activity simply as a percentage of the total Chloramphenicol acetylated.

In this study, the T helper cell lines were activated by various stimuli following transfection. Expression of the META element appeared to be absolutely dependent on activation of the transfected T-helper cell. Therefore, given the fact that viability and recovered cell numbers varied depending on whether or not the cells were activated, use of equivalent amounts of total protein in the CAT assay was considered appropriate for this study. Although the cells may no longer be viable, protein may accumu-

late to the highest levels in the cells which have been activated to the highest extent i.e., the ones which are most likely to undergo apoptosis. Therefore, there is no perfect basis for comparison and no absolute standard, only relative responses with adequate controls.

Viral promoters such as the RSV LTR, SV40 early region, and herpes simplex tk are used to drive both expression of CAT and other reporter genes. Discussions with colleagues and review of the literature indicated that although these are considered to be constitutive promoters, activity may vary between cell types, species of origin, and state of activation. It has recently been documented that expression of pRSV-Cat and pSV2-Cat can vary both with the activation state and the differentiation stage of the transfected T cell (145). In the studies presented in this chapter cells from different species and in different states of activation were utilized. Therefore, in these preliminary studies neither an internal control nor normalization of the CAT activity data relative to activity obtained from an additional reporter plasmid [such as pRSV(cat) or ptk(cat)] were used.

Assay for IL2

Supernatant media were collected from activated T cell cultures at the time of harvest and stored at -20°C until tested. The IL2 assay was performed as described previously (91) using the IL2-dependent cell line MTL2.8.2, and monitoring the reduction of the tetrazolium dye MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide [Sigma]) (140). CsA has no effect on this assay. The IL-2 assay was initially standardized relative to a reference standard from Biological Resources Branch, NCI-FCRF (Frederick, MD). Half-maximal stimulation of T cell lines used in this work occurred between 1-5 U/ml IL2.

RNA Isolation

Total cellular RNA was isolated by a modification of the guanidine thiocyanate-cesium chloride gradient method (26, 170). The RNA isolation reagent was prepared by gently heating guanidine thiocyanate (final concentration 4 M), sodium N-lauroylsarcosine (final concentration 0.5%), and sodium citrate (final concentration 2.5 mM). The solution was adjusted to pH 7.0 and filtered to remove particulate matter. On the day of use, 2-mercaptoethanol was added to achieve a final concentration of 0.1 M.

Up to $30x10^6$ cells were washed in PBS and pelleted. The pellet was resuspended in 3 ml of guanidine thiocyanate solution and homogenized by aspiration 5 to 6 times through a 21g needle attached to a 5 ml disposable syringe. The samples were stored at -70 C until processed. The RNA sample was layered over 2 ml of cesium chloride (5.7 M CsCl, 0.01M EDTA, pH 7.0) and spun for up to 18 hr using a Beckman SW50.1 rotor at 36,000 rpm. The RNA pellet was resuspended in Tris-EDTA buffer (TE, pH 8.0: 10 mM Tris-HCl, 1 mM EDTA) and precipitated by addition of a one tenth volume of 3 M sodium acetate-pH 5.2 and two volumes of ethanol. The precipitate was washed twice in 70% ethanol, resuspended in TE, and quantitated using ethidium bromide fluorimetry (139).

Northern Analysis

Up to 20 µg of RNA was denatured for 15 min at 55°C in a solution containing 50% formamide, 6.5% formaldehyde and 1X MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0). The RNA was size fractionated for 4 hr at 120V through a 1% agarose gel containing 0.67% formaldehyde and 0.33 µg/ml ethidium bromide in a BRL electrophoresis gel box modified to allow circulation of the MOPS running buffer. RNA was transferred by capillary action onto nylon membrane (Hybond-N, Amersham). The gel was placed face-down on a clean glass plate followed by the nylon membrane and two sheets of Whatman 3MM paper presoaked in 10x SSC (1X SSC, pH 7.0: 150 mM NaCl, 15 mM sodium citrate). The pile was completed by adding 2 sheets of dry filter paper, two inches of paper towel (all components were cut to the size of the gel), a glass plate and a 1 kg weight. The structure was left for at least 12 hr and the RNA was cross-linked to the support using a UV Stratalinker™ for the recommended exposure.

Filters were prehybridized for 8 to 16 hr in solutions containing:

For DNA fragments; 50% formamide, 5X SSC, 5X Denhardt's solution (100X Denhardt's: 1% Pharmacia Type 400 Ficoll, 1% polyvinylpyrrolidone, 1% BSA), 0.1% SDS, and 200 µg/ml denatured, sheared salmon sperm DNA.

For oligonucleotide probes; 6X SSC, 5X Denhardt's solution, 0.1% SDS.

Hybridizations were carried out in the same buffers for 12 to 18 hr at temperatures indicated in individual experiments. Generally hybridizations

were done at 42°C for DNA fragments and three to five degrees below the Tm for oligonucleotide probes. Blots were washed as indicated in individual experiments.

Primer Extension

The primer extension assay was performed essentially as described elsewhere (170). Experimental conditions including; amount of radiolabeled primer, type of hybridization buffer, hybridization temperature and length of hybridization were varied in a number of experiments. Hybridization incubation times were varying between 1 and 24 hr and it was determined that an minimum of 6 hr appeared to be required. Experimental conditions were selected which resulted in the lowest background and maintained a reproducible pattern of primer extension products. Briefly, oligonucleotide primers Mtv.A08 and Mtv.A16 (Table 2-1) were end-labeled with ^{32}P then 10 µg of total RNA was mixed with $5x10^4$ cpm of labeled oligonucleotide probe ($2.4x10^7$ cpm/µg) in hybridization buffer which consisted of 40 mM PIPES-pH 6.4, 1 mM EDTA-pH 8.0, 0.4 M NaCl, and 80% formamide. The nucleic acids were denatured by heating to 85°C for 10 min and were then transferred to a 45°C water bath for overnight hybridization. The sample was precipitated by the addition of 170 µl water and 400 µl ethanol. After incubation on dry ice for one hr, samples were spun at 14,000 rpm in an Eppendorf microfuge at 4°C. The pellet was washed once with 70% ethanol and dried briefly. It was important not to over-dry the sample because it was very difficult otherwise to resuspend the RNA/primer in the reverse transcriptase buffer. The final 30 µl reverse transcription reaction mixture contained 50 mM Tris-pH 7.6, 60 mM KCl, 10 mM $MgCl_2$, 500 µM of each dNTP, 1 mM dithiothreitol, 1 µl RNasin (placental RNase inhibitor obtained from Promega), and 200 U cloned Moloney murine leukemia virus reverse transcriptase (Gibco BRL). It was found that incubation for one hr at 42°C resulted in less nonspecific cDNA products when compared to incubation at 37°C. Following reverse transcription, 150 µl of TE/sodium acetate was added to achieve a final concentration of 0.3 M sodium acetate. The samples were extracted once with chloroform-phenol, ethanol precipitated, and the pellet was resuspended in 4 µl of TE and 6 µl of formamide loading buffer (80% formamide, 10 mM EDTA, 1 mg/ml xylene cyanol FF, 1 mg/ml bromophenol blue). After heating for 5 min at 95°C, radiolabeled cDNA was

Table 2-1. Oligonucleotides primers

Oligonucleotide	Sequence	Restriction Site	Position
Mtv.A01	gccgcaTGCCGCAGTCGGCCGACCTGA	Sph I	9901
Mtv.A08	CTCTCGGGAGTTCAACCATTTCTG	None	8611
Mtv.A16	GGtcaGGGAAGGCGCAAGGCAACC	None	7328
Mtv.A11	CTTCggaTccATgtcGACCAGTTTTGTATTGGCC	BamH I, Sal I	7212
Mtv.S02	GccGAgCTCCGAAGCGGAGGAGGT	Sac I, Sst I	6750
Mtv.S11	GACCggATccCaaGCTTTGTTGTCTGTCCTCGG	BamH I, Hind III	6814
MIL2.A01	GATGATGCTTTGACAGAAGGCTATCCATCT	None	
HIL2.A15	TGTTGAGATGATGCTTTGACA	None	

The primers sequences are shown 5' to 3'. They are either sense (Mtv.Sxx) or antisense (Mtv.Axx), relative to the MMTV map. Some of the nucleotides were changed from the known MMTV sequence at their 5' ends, to provide restriction sites (mismatches are indicated in lower case letters, restriction sites are underlined). The nucleotide written in bold for each primer corresponds to the MMTV map position given in the last column (numbering of Moore et al. 1987, Ref 138). MIL2.A01 and HIL2.A15 are antisense oligonucleotides taken from the mouse and human IL2 cDNA sequences respectively.

separated under denaturing conditions on a 7 M urea-6% acrylamide gel and visualized by autoradiography. End-labeled denatured fragments generated from pGEM2 DNA cut with Hinf I and PM2 DNA cut with Hae III were used as markers.

RNase Protection Assay

Radiolabeled RNA was synthesized using reagents and recommended protocol supplied within the Promega Riboprobe System (192). The plasmids pCM1 and pCM2 (Figure 2-3) were linearized with Sal I and EcoR I respectively, extracted once with phenol-chloroform and ethanol precipitated. The 20 µl in vitro transcription reaction contained, 0.3 µg template DNA, transcription buffer (5X transcription buffer: 200 mM Tris-HCl-pH 7.5, 30 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl), 10 mM dithiothreitol, 20U rRNasin ribonuclease inhibitor, 0.5 mM each of ATP, GTP, and UTP, 6 µM CTP and 12 µM [α-^{32}P] CTP (specific activity, 800 Ci/mmol) and 15u T7 or SP6 RNA polymerase (pCM1 and pCM2 respectively). Following incubation for 1 hr at 37°C or 40°C (when using T7 or SP6 RNA polymerase respectively), the template DNA was digested with DNase and the unincorporated nucleotides were removed by separation through a G-50 spin column (170). The column eluate was precipitated with sodium acetate/ethanol and the radiolabeled RNA was resuspended in hybridization buffer (80% formamide, 40 mM PIPES-pH 6.4, 0.4 M NaCl, 1 mM EDTA). Total RNA was combined with $5X10^5$ cpm of RNA probe in hybridization buffer, heated for 5 min at 85°C and hybridized at 45°C for 6 to 12 hr. Non-hybridized probe was digested with 40 µg/ml of RNase alone or in combination with 367 U/ml of Ribonuclease T1 (Gibco BRL) in 10 mM Tris-HCl-pH 7.5, 5 mM EDTA and 300 mM NaCl for 60 min at 30°C. The reaction was terminated by incubation with 0.5% SDS and 0.27 mg/ml of proteinase K. Following chloroform-phenol extraction and ethanol precipitation, the sample was resuspended in formamide loading buffer (80% formamide, 10 mM EDTA, 1 mg/ml xylene cyanol FF and 1 mg/ml bromophenol blue) heated to 95°C for 10 min and separated on a denaturing 4.8% acrylamide-7 M urea gel.

Radioactive labeling of DNA fragments and oligonucleotides

DNA fragments were labeled using an adapted random primer method (55). The 5X random primer solution was made up by combining

100 parts A (A: 0.5 mM each of dATP, dTTP and dGTP in 1.25 M TRIS-HCl, 0.125 M $MgCl_2$, pH 8.0, 0.25 M 2-mercaptoethanol), 250 parts B (B: 2 M HEPES, pH 6.6) and 150 parts C (C: hexadeoxyribonucleotides [$pd(N)_6$ obtained from Pharmacia, product no. 27-2166, Lot no. 97899] at 90 OD U/ml in TE). For the labeling reaction up to 1 µg of DNA in TE was heated for 5 min at 95°C, cooled on ice and the following reagents added to achieve a final volume of 50 µl; 10 µl 5X random primer solution, 20 µg bovine serum albumin (Boehringer Mannheim), 200 mCi α^{32}P-dCTP (specific activity, 800 Ci/mmol) and 3U Klenow (GIBCO BRL). The reaction was incubated at room temperature for 3 to 18 hr.

Oligonucleotides were ^{32}P end-labeled using T4 polynucleotide kinase (37). Up to 300 ng of oligonucleotide was incubated in kinase buffer (10X kinase buffer: 500 mM Tris pH 7.4, 100 mM $MgCl_2$, 50 mM DTT and 10 mM spermidine) with 10U T4 polynucleotide kinase and 200 µCi γ-^{32}P-ATP (specific activity 3000 Ci/mmol) in a 50 µl total volume. The reaction was incubated for 30 min at 37°C and the enzyme was inactivated by heating to 65°C for 5 min.

Following both labeling techniques, unincorporated label was removed using spin columns (170). For this procedure, a disposable 1 ml syringe barrel was plugged with a small amount of siliconized glass wool and filled with Sephadex G-50 (medium) equilibrated in TE, pH 8.0. The bed resin was washed with a further 2 ml of TE. A microcentrifuge tube with the cap removed was placed within a 15 ml culture tube and the syringe column was inserted such that the tip rested within the microfuge tube. The column was spun for 2 min at 1200 rpm and the sample was loaded onto the top of the column in a 50 µl volume. The column was re-spun under the same conditions and the labeled oligonucleotide or DNA fragments were used without further purification.

Synthesis of oligonucleotides and DNA sequencing

Oligonucleotides used in the study were synthesized either in the Department of Biochemistry using an Applied Biosystems 392 DNA/RNA synthesizer or in the Department of Microbiology using model ABI 391. The reactions for DNA sequencing were performed by Rosemary Garner using the protocol and reagents recommended in the Sequenase Version 2 sequencing kit (USBiochemical Co.). The sequencing gels were run by Perry

d'Obrenan and/or Rita Whitford using facilities in the Department of Biochemistry, University of Alberta. The MacVector 3.5 computer program was used for analyzing and handling of DNA sequence information.

Isolation of Genomic DNA

Genomic DNA was isolated from EL4.E1 cells, 12.1.19 cells and BALB/c splenocytes (37). Cells were washed in PBS and one volume of packed cells was incubated with ten volumes of proteinase K solution (1 mg/ml proteinase K, 10 mM Tris-pH 7.4, 10 mM EDTA, 150 mM NaCl and 0.4% sodium dodecyl sulfate) for 15 min at 65°C and then overnight with gentle shaking at 37°C. The solution was extracted 3 or 4 times using phenol: chloroform (1:1) and DNA was precipitated from the aqueous phase using 1/10 volume of 3 M sodium acetate-pH 5.2 and 2.5 volumes of ethanol. The DNA pellet was washed twice in 70% ethanol and resuspended in TE.

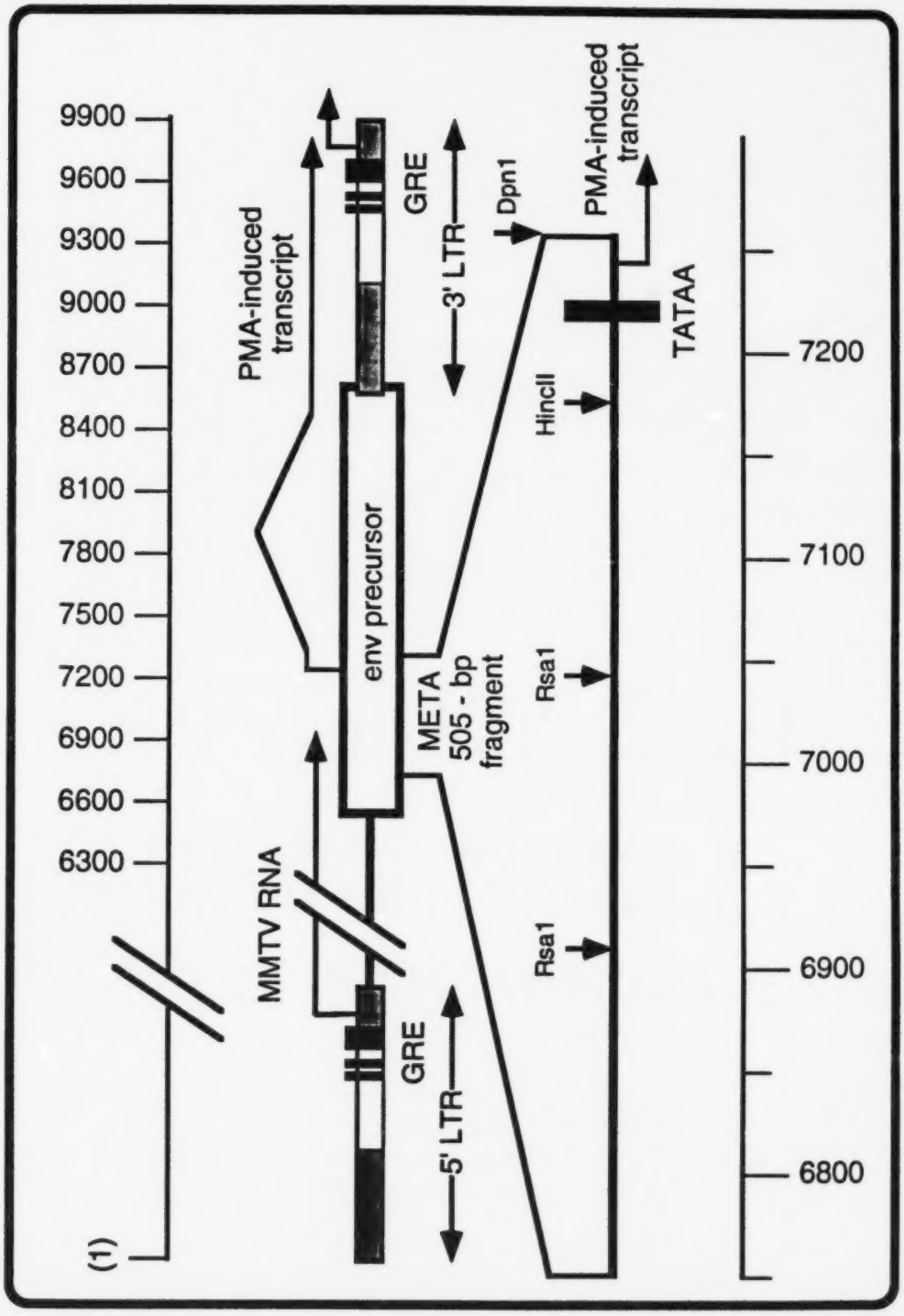
Isolation of MMTV fragments

MMTV proviral sequences were isolated from genomic DNA of EL4.E1 cells, 12.1.19 T hybridoma cells, or from primary BALB/c spleen cells using the polymerase chain reaction (PCR). The 100 µl reaction contained 500 ng genomic DNA, 0.25 µM each of the upstream and downstream primers, 200 µM each of the αNTPs, 50 mM KCl, 10 mM Tris-HCl-pH 8.3, 2.5 mM $MgCl_2$, 0.01% gelatin and 2.5 U Taq polymerase (BRL). Samples were overlaid with two drops of mineral oil and processed in a Hybaid™ thermal reactor (Bio/Can Scientific, Mississauga, Ontario). PCR conditions were; one cycle at 94°C for 3 min, 25 cycles consisting of; 1 min denaturation at 94°C, 1 min annealing at 55°C and 1.5 min extension at 72°C, and a final extension at 72°C for 5 min.

The MMTV sequence between 6750 and 9901 (Figure 2-1) was copied from DNA of EL4.E1 or 12.1.19 cells using the upstream (sense) primer Mtv.S02 and the downstream (antisense) primer Mtv.A01 (Table 2-1). All MMTV sequences are numbered according to the milk-borne virus (138) with position 1 corresponding to the first nucleotide of the 5'LTR. DNA from BALB/c spleen was copied using the same upstream primer, and the downstream primer Mtv.A16, which ends at position 7333. The PCR product from 12.1.19 cells was of length 3.2 kb, whereas the EL4.E1 copies were 494 bp shorter due to the deletion within the 3' LTR (51).

Figure 2-1. Relevant features of the MMTV genome in EL4.E1 cells

The 3' portion of the map contains the sequence encoding the *env* protein precursor mRNA and the 3' LTR. Conventional full-length transcription initiates near the 3' end of the 5' LTR ("MMTV RNA"). In the EL4.E1 T lymphoma cell line, the LTR (stippled box) contains a 494 base-pair deletion, shown by the white box within its central portion. This deletion removes a part of the glucocorticoid response elements (GRE). In EL4.E1 cells the novel promoter in the envelope gene initiates a transcript just downstream of a canonical TATA box. The transcript initiated in the *env* gene is spliced as shown (51). The 505 bp segments amplified and cloned from the MMTV *env* transcriptional activator (META) derived from EL4.E1 and from 12.1.19 cells are represented by the fragment indicated. The META fragment isolated from BALB/c spleen cells is 583 bp in length and extends from position 6750 to position 7328. Restriction enzyme cleavage sites for Rsa I, Hinc II and Dpn I are indicated.


9900
9600
9300
9000
8700
8400
8100
7800
7500
7200
6900
6600
6300
(1)
MMTV RNA
GRE
5' LTR
PMA-induced transcript
env precursor
META
505 - bp fragment
GRE
3' LTR
Dpn1
HincII
Rsa1
Rsa1
PMA-induced transcript
TATAA
7200
7100
7000
6900
6800

Nomenclature used for MMTV fragments and recombinant plasmids

The recombinant plasmids and MMTV fragments are identified using the conventions illustrated by the following example:

pGEM(cat)C30(6750/7255)

pGEM(cat)-refers to the parental plasmid into which the various MMTV fragments were inserted

C30- refers to a specific MMTV cloned sequence, in this case generated by PCR from EL4.E1 genomic DNA

(6750/7255) - the numbers in parenthesis refer to the nucleotide at each end point of the MMTV fragment

The sequence is numbered using as reference the BR6 milk-borne sequence published by Moore et al (138). In some cases, the cloned sequences contained deletions or insertions relative to the milk-borne. In these cases, numbering still reflects the published milk-borne sequence.

The designation (7255/6750) would refer to the same fragment described in the above example, but in the reverse orientation.

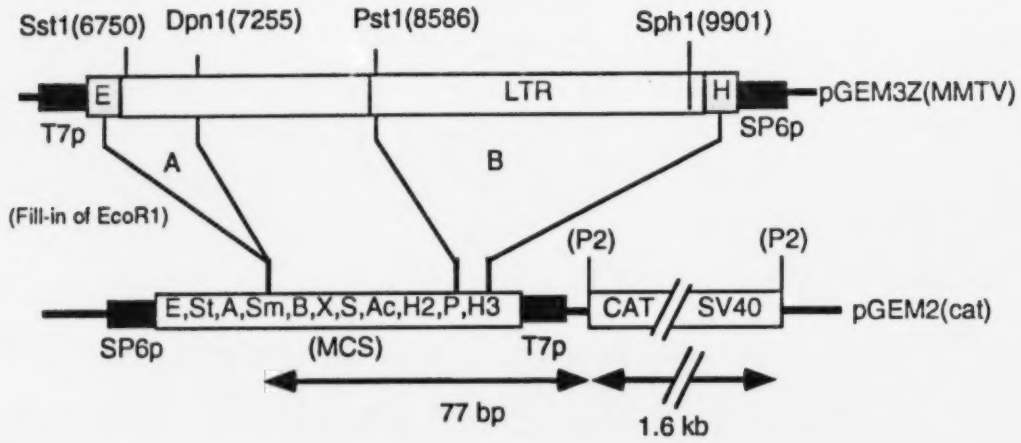
MMTV recombinant plasmids

The pGEM(cat) vectors were constructed by ligation of the 1.6 kb Hind III-BamH I fragment from pSV2(cat) (76), which includes the bacterial Chloramphenicol acyltransferase gene and the small T intron and polyadenylation signal from SV40, into the Pvu 2 site of pGEM1 or pGEM2 (Promega) (63). The vector pBLCAT2, contains the herpes simplex virus thymidine kinase (tk) promoter upstream of the CAT coding sequence (124). pBLCAT3 is analogous to the pBLCAT2 except that it does not contain the tk promoter. The plasmid pRSV(cat) (75) was used in all experiments as a control representing a constitutively active promoter construct.

The initial PCR products, generated from EL4.E1 and 12.1.19 genomic DNA, were cut with the relevant restriction enzymes (as indicated in Table 2-1) and cloned into the plasmid pGEM3Z (Promega Corp.) which had been cut with Sst I and Sph I. After cloning, *env* fragments covering the MMTV sequence from 6750 to 7255 were excised from the pGEM3Z recombinant with EcoR I (which lies just to the 5' side of the Sst I site in pGEM3Z) and Dpn I (MMTV sequence position 7255, see Figure 2-1 and Figure 2-2). The fragments were treated with Klenow DNA polymerase to fill in the EcoR I

Figure 2-2. General structure of the pGEM2(cat)-MMTV recombinant plasmids

Construction of the vectors is described in the section titled 'MMTV recombinant plasmids'. The 1.6 kb fragment containing the CAT coding region and the SV40 splice and polyadenylation regions was inserted into the Pvu II site of pGEM2 to obtain pGEM2(cat). A: 505 bp fragments, derived from EL4.E1 and 12.1.19 cells, were excised from pGEM3Z(MMTV) clones and were blunt-ended prior to ligation into the Sma I site of pGEM2(cat). A 583 bp fragment was generated by PCR from BALB/c spleen DNA. This fragment extends from position 6750 to 7328 and was directionally cloned into pGEM2(cat) cut with Sst I and Sma I. The coding sequence of the CAT gene is 77 bp from the Sma I site. B: The LTR portions of the pGEM3Z-(MMTV) clones were isolated by cleavage with Pst I and Hind III and insertion into the corresponding restriction sites in pGEM2(cat). Restriction sites: A-Ava I, Ac-Acc I, E - EcoR I, B - BamH I, H2 - Hinc II, H3 - Hind III, P - Pst I, P2 - Pvu II, R - Rsa I, S - Sal I, Sm - Sma I, Sp - Sph I, St - Sst I/Sac I, X - Xba I. Other symbols: MCS - multiple cloning site; T7p, SP6p - T7 and SP6 promoters.


Sst1(6750)
Dpn1(7255)
Pst1(8586)
Sph1(9901)
E
LTR
H
pGEM3Z(MMTV)
T7p
SP6p
A
B
(Fill-in of EcoR1)
(P2)
(P2)
E,St,A,Sm,B,X,S,Ac,H2,P,H3
CAT
SV40
pGEM2(cat)
SP6p
(MCS)
T7p
77 bp
1.6 kb

sites, and blunt-end ligated into the Sma I site of pGEM1(cat) or pGEM2(cat) (63). The LTR segments were excised from the pGEM3Z recombinants with Pst I (MMTV position 8586) and Hind III (in the pGEM3Z multiple cloning site), and cloned into pGEM2(cat) which had been treated with the same restriction enzymes (Figure 2-2). The MMTV fragment copied from BALB/c splenic DNA extended from positions 6750 to 7328. It was inserted into pGEM2(cat) DNA which had been cut with Sst I and Sma I. The cloned sequences C30, C23 and C32 were derived from EL4.E1 DNA. C11 was from 12.1.19 cellular DNA, and 15-8 was from BALB/c spleen cells.

Shorter fragments of the MMTV region were also generated by cleaving the original 505-bp inserts with either Rsa I or Hinc II (cleavage at positions 7002 and 7172, respectively). These fragments were inserted into the multiple cloning site (MCS) of pGEM1(cat) or pGEM2(cat).

MMTV fragments, for insertion into pBLCAT2 and pBLCAT3 were PCR copied from the primary MMTV clones using sense primer Mtv.S11 (position 6814) and antisense primer Mtv.A11(Table 2-1). This antisense primer begins copying the MMTV sequence at 7212, and deletes the TATA box and the starting point for clone 14 transcription (51) which occurs downstream from it. The shortened PCR product was cleaved with BamH I and inserted into pBLCAT2 or pBLCAT3 which were similarly prepared.

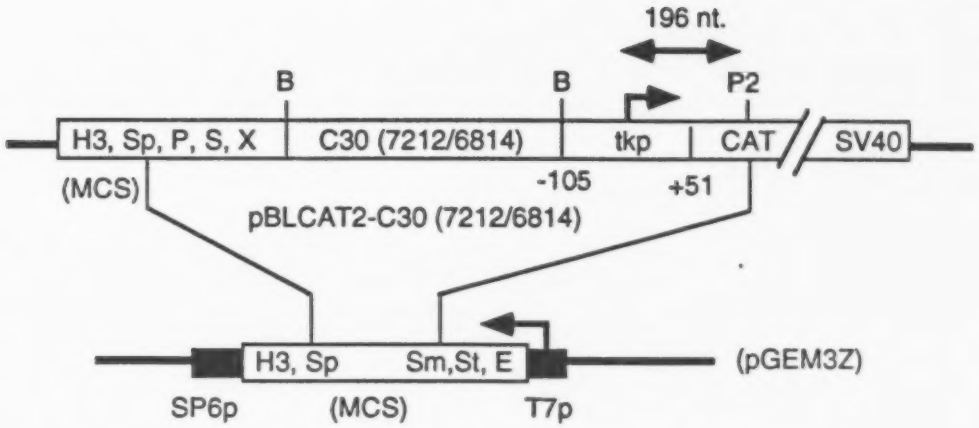
The plasmids pCM1 and pCM2, were constructed to generate the RNA probes used in RNase protection experiments (Figure 2-3). For pCM1 the plasmid pBLCAT2-C30(7212/6814) was cut within the MCS with Sph I and within the CAT coding region with Pvu 2. The resulting fragment, approximately 720 bp in length was ligated into pGEM3Z which had been digested with Sph I and Sma I. The recombinant plasmid produced contained the MMTV sequences (7212/6814) upstream of the 156-bp *tk* promoter, which in turn was followed by the CAT coding sequence (Figure 2-3, panel A). pCM2 was constructed by cleavage of pGEM2(cat)C30(6750/7255) with Sst I and Pvu 2 and insertion of the resulting fragment into pGEM1 which had been treated with Sst I and Sma I(Figure 2-3, panel B).

Preparation of Plasmid DNA

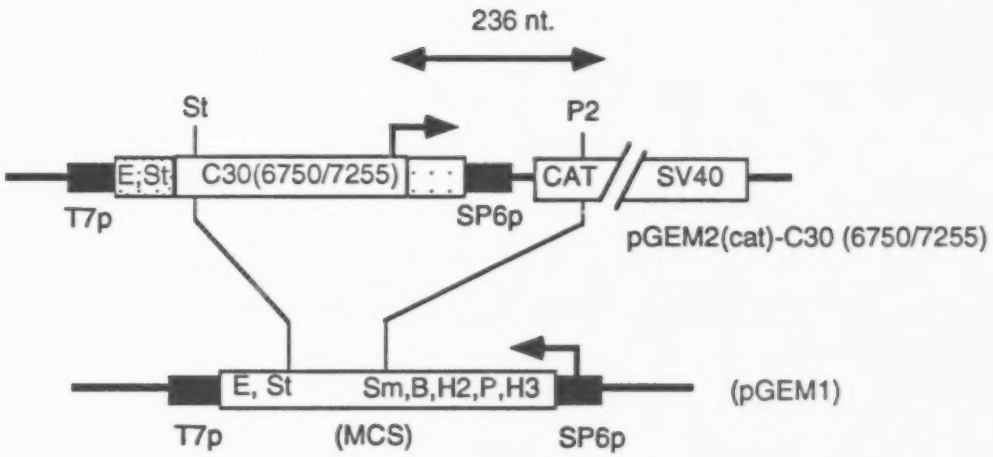
DNA fragments used in the cloning procedures were isolated from agarose gel slices using either a method described by Heery (89) or by glass powder elution (37). Smaller fragments (less than 300 bp) were separated by

Figure 2-3 Structure of pCM1 and pCM2

pCM1 and pCM2 plasmids were created to serve as template for synthesis of radiolabeled RNA for use in RNase protection experiments. Construction of the plasmids is described in the text. For pCM1 shown in A: the META fragment [C30(7212/6814)] does not contain a TATA box (position 7215), and is in the reverse orientation, relative to the MMTV genome. The starting site for transcription within the tk promoter (tkp) is indicated, and lies 51 bp upstream of the starting point of the CAT coding sequence. The segment indicated was transferred to pGEM3Z, where T7 RNA polymerase was used to copy it. Only 196 nucleotides of the resulting RNA probe should be protected by transcripts which initiate in the tk promoter. For pCM2 shown in B: The arrow within the segment C30(6750/7255) indicates the endogenous META transcript start site at position 7247. The multiple cloning site within pGEM(cat)C30(6750/7255) is indicated by a stippled box and restriction enzyme designation is provided in legend to Figure 2-2. Antisense RNA probe is generated from pCM2 using SP6 RNA polymerase. In the RNase protection assay, a protected fragment 236 nucleotides in length would be expected if RNA containing the CAT coding sequence initiated at position 7247 within C30(6750/7255).



A. Structure of plasmid pCM1



B. Structure of plasmid pCM2

polyacrylamide gel electrophoresis and recovered using the "crush and soak procedure"(170). Vector preparation and cloning procedures were carried out according to published protocols (37). Following the ligation step, a portion of the ligation mixture was diluted four fold in TE, transformed into Escherichia *coli* DH5α (Gibco/BRL), and plated on LB (Luria-Bertani) plates containing 100 μg/ml ampicillin. Recombinants were screened by picking individual colonies for growth in 2 ml LB/ampicillin broth cultures and small scale plasmid preparations were done using the alkaline lysis method (14). Desired recombinants were identified by restriction enzyme analysis and verified by double-stranded DNA sequencing.

Large scale plasmid DNA was prepared using the alkaline lysis method (170). As required, plasmids were purified by centrifugation in cesium chloride-ethidium bromide density gradients (170). When use of high purity closed circular plasmid was not crucial, DNA, from the alkaline lysis procedure was resuspended in TE buffer, treated for 30 min with 30 μg/ml of RNase A (Boehringer-Mannheim) and for a further one hour with 50 μg/ml proteinase K (Boehringer-Mannheim). The sample was then deproteinized by two extractions using an equal volume of chloroform-phenol (1:1) followed by one chloroform extraction (1:1). DNA was precipitated with 1/10 volume of 3M sodium acetate-pH 5.2, and two volumes of 95% ethanol. The sample was stored at -20°C for at least an hr. Following centrifugation for 30 min at 10,000 rpm, the DNA was washed twice with 70% ethanol, dried and resuspended in an appropriate volume of TE. DNA was quantitated by ethidium bromide fluorimetry (139).

CHAPTER THREE. TRANSCRIPTION IN PMA-TREATED EL4.E1 CELLS

Introduction

The mouse lymphoma cell line EL4.E1, which is a variant of the EL4 line (54) synthesizes a number of cytokines including IL2, IL5, GM-CSF and IL10 in response to PMA stimulation. Previous work from this laboratory demonstrated that PMA also induced an increase in mRNA of several non-cytokine genes including a proteoglycan core protein, serglycin (49), and an abundant set of transcripts corresponding to sequences present in the 3' region of MMTV (73% of the 136 randomly-selected cDNA clones) (51).

The MMTV transcripts appeared to initiate within the *env* gene of chromosomally integrated MMTV. Accumulation of the transcripts was inhibited by CsA in a pattern analogous to that seen for several cytokine genes. Sequence analysis of a prototypic cDNA clone (clone 14) and determination of the mRNA "cap" site localized the transcriptional start site within the *env* gene. Initiation occurred about 30 nt downstream from a TATAA element positioned at 7215 and an intron 1161 bp in length, extending from position 7339 to 8500, was spliced out resulting in a mature mRNA approximately 0.9 kb in size. The splice acceptor site is the same one used in the generation of the LTR transcript from the conventionally transcribed, full-length MMTV RNA (138). These results were surprising in that MMTV transcription normally arises within the conventional promoter/enhancer present in the 5' LTR.

The PMA-inducible mRNA includes the open reading frame (*orf*) present within the MMTV 3' LTR. The *orf* gene corresponds to the Mls loci, and the encoded proteins appear to function as murine superantigens (2). These experiments suggested that a novel promoter/enhancer exists within the MMTV *env* gene in EL4.E1 cells and that transcription is responsive to the same signals which induce or suppress cytokine gene expression. This putative promoter/enhancer will be referred to as the MMTV *env* transcriptional activator (META).

It was hypothesized that the META element is a lymphocyte-specific promoter/enhancer, responsive to activation signals in T helper cells with the capacity to transcribe the Mls loci. If so, characterization of it may be

important for the understanding of mechanisms of self-tolerance and MMTV-induced lymphomagenesis. The aim of preliminary studies was to further characterize META transcription in EL4.E1 cells. Data will be presented which confirm that META transcripts initiate within the *env* gene of endogenous MMTV in activated EL4.E1 cells and that induction and suppression correlate with IL2 gene expression. During studies aimed at characterization of cell lines that would be suitable for the proposed transfection experiments, it was found that induction of IL2 production from a T-cell hybridoma, 12.1.19 cells also resulted in accumulation of mRNA for the proteoglycan core protein, serglycin (49).

META transcripts are induced in EL4.E1 cells treated with PMA

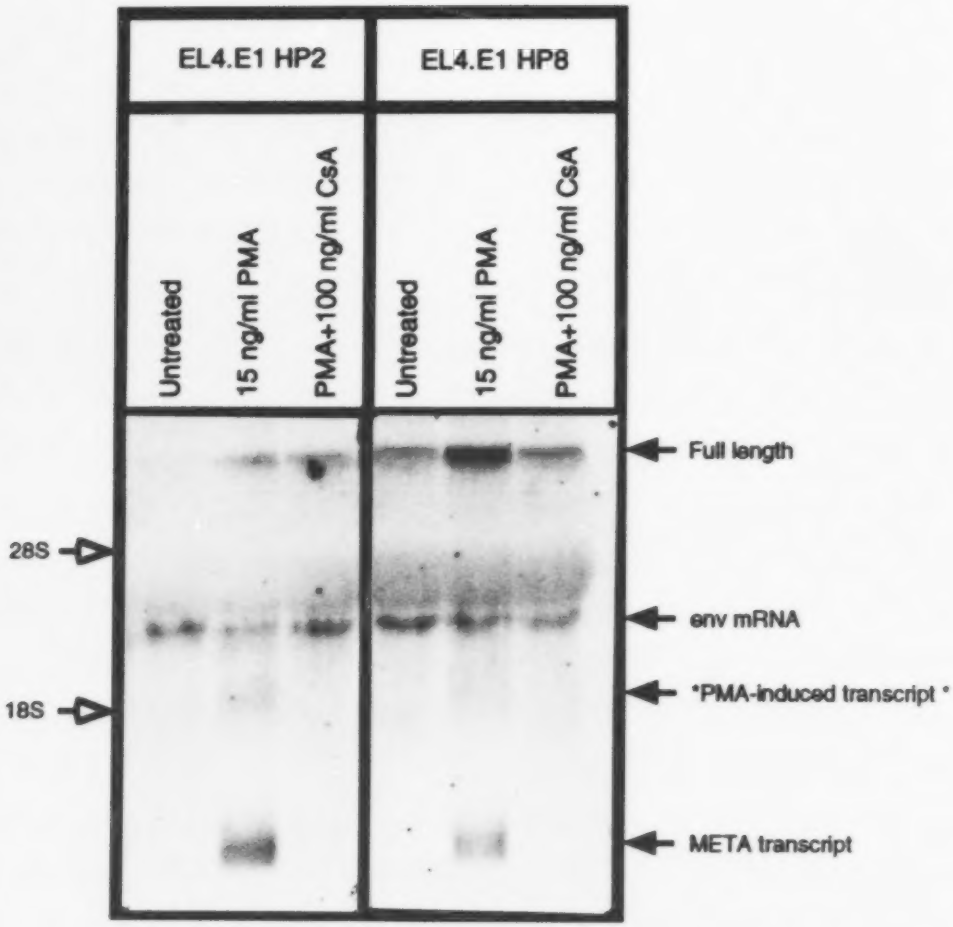
The EL4.E1 line was recloned and two isolates, EL4.E1 HP2 and EL4.E1 HP8 were selected based on their capacity to produce high levels of IL2 in response to treatment with 15 ng/ml of PMA. Preliminary studies were done to characterize META transcription in these subclones. Cells set up at a density of $5x10^5$/ml were separated into three groups; untreated, 15 ng/ml PMA and 15 ng/ml PMA + 100 ng/ml CsA. Following incubation for 12 hr, the cells were harvested, washed and total cellular RNA was isolated. Northern analysis was performed as described in Chapter 2 using ^{32}P-end-labeled oligonucleotide Mtv.A16 whose 5' nucleotide lies at position 7333 within the *env* gene (Table 2-1). Results are shown in Figure 3-1.

The full-length transcript and env mRNA, which both initiate at position 1195 in the 5' LTR, are shorter than normal (51, 155) because of the 494 bp deletion in the LTR of amplified MMTV provirus in EL4.E1 cells (51,115). Neither PMA nor a combination of PMA and CsA appreciably altered the detected levels of full-length or env mRNAs when compared to untreated cells, whereas META transcripts were significantly increased by induction and accumulation was blocked by CsA.

The identity of the additional PMA-inducible, CsA-suppressible transcript detected near the 18S rRNA marker is unknown. The probe used would not detect the 1.85 kb transcript described by Kwon and Weissman (115) nor the 1.7 kb LTR-ORF mRNA (138, 154). It has been reported that EL4 cells synthesize a 1.85 kb env mRNA generated by alternate spicing but transcription was not dependent on phorbol ester treatment (155).

Figure 3-1 Induction of META transcription in PMA-treated EL4.E1 cells

EL4.E1 HP2 and EL4.E1 HP8 were set up at a density of $5x10^5$/ml and cultures were separated into three treatment groups; untreated, 15 ng/ml PMA and 15 ng/ml PMA + 100 ng/ml CsA. Following incubation for 12 hr, the cells were harvested, washed once in PBS and total cellular RNA isolated. The RNA was fractionated through a 1% agarose-formaldehyde denaturing gel and Northern analysis was performed as described in Chapter 2. The oligonucleotides Mtv.A16 and Mtv.S02 (Table 2-1) were end-labeled with ^{32}P using T4 kinase to a specific activity of $4.3x10^7$cpm/µg and $4.4x10^7$cpm/µg respectively. The RNA filter blot was prehybridized for 4 hr at 56°C and hybridized for 6 hr at 56°C using Mtv.A16. Final washes were done at 45°C in 6XSSC, 0.1% SDS. An additional PMA-induced transcript is indicated by (**). The results using Mtv.S02 are discussed in Chapter 4.


EL4.E1 HP2
EL4.E1 HP8
Untreated
15 ng/ml PMA
PMA+100 ng/ml CsA
Untreated
15 ng/ml PMA
PMA+100 ng/ml CsA
28S
18S
Full length
env mRNA
*PMA-induced transcript *
META transcript

META transcription parallels that of the IL2 gene

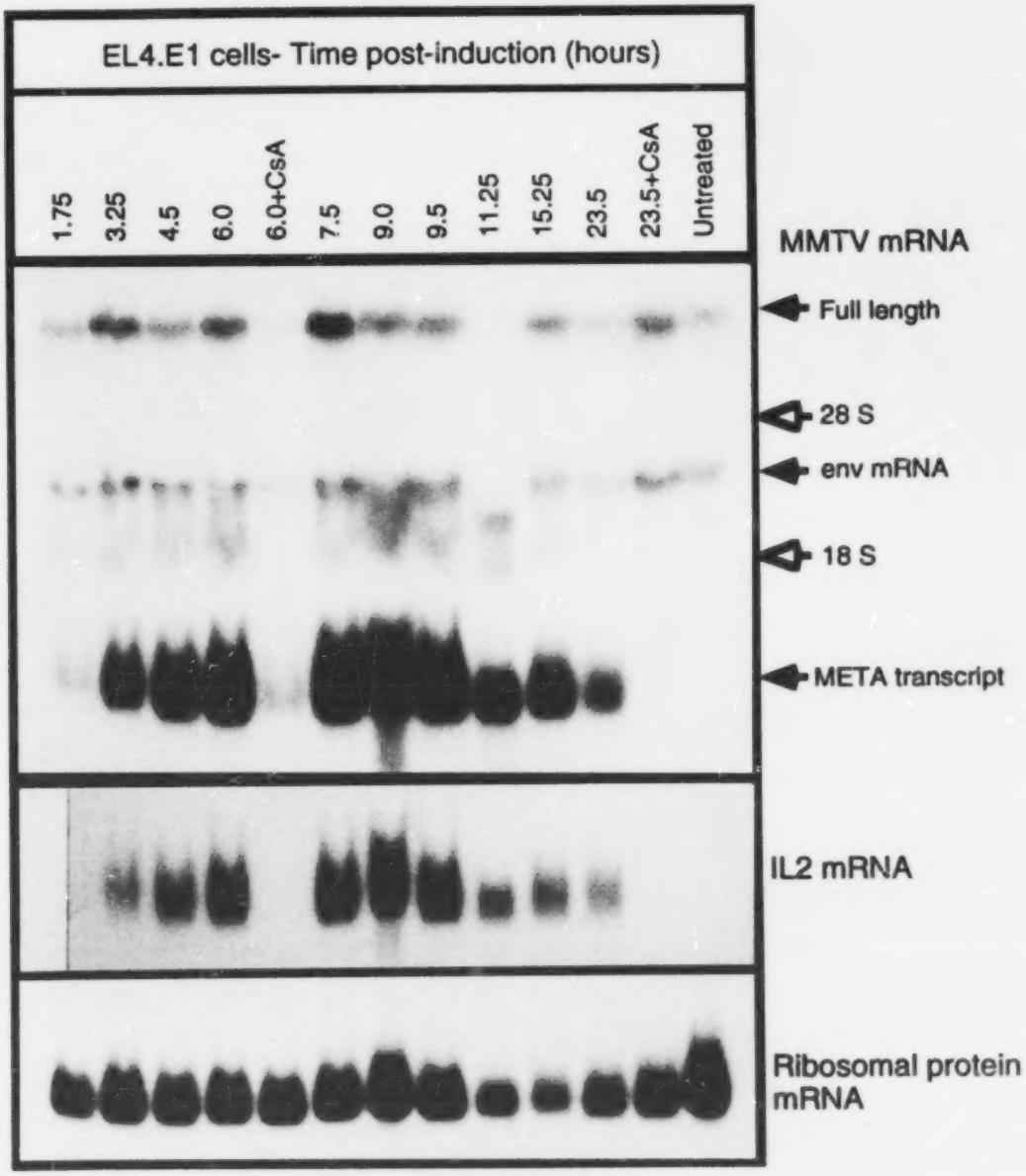
The pattern of induction and suppression by CsA of IL2 mRNA and META transcripts in PMA-treated EL4.E1 cells is similar (176, 51). In order to further examine this relationship, RNA was isolated at various intervals following activation of EL4.E1 cells (Figure 3-2). EL4.E1 HP2 cells were treated with 15 ng/ml PMA and 1.5 μM ionomycin for different times prior to the isolation of total RNA. Ionomycin in combination with PMA has been shown to enhance IL2 production from T-cells (195). Two samples also contained 100 ng/ml of CsA. The Northern blot was probed with ^{32}P-labeled oligonucleotide, MIL2.A01 (Table 2-1).

IL2 mRNA was detectable by 3.25 hr post-induction and accumulation reached a plateau between 7.5 and 9.5 hr. The blot was reprobed with a MMTV LTR oligonucleotide, Mtv.A08 (Table 2-1). The META transcript, when compared to ribosomal protein RNA, was detectable by 1.75 hr and continued to accumulate until at least 7.5 hr post-induction. It is not known whether the decline in abundance of META transcripts after about 15 hr post-induction is due to an altered transcriptional rate or a change in RNA stability. CsA added at the outset inhibited both META and IL2 gene expression. It is difficult to evaluate whether PMA treatment resulted in an increase in transcription from the 5' LTR. Several samples showed evidence of non-proportional RNA sample loading when compared to levels of mRNA encoding a ribosomal protein of the L32 gene family (44).

In summary PMA treatment of EL4.E1 cells resulted in a rapid accumulation of the META transcript and the kinetics of induction and suppression by CsA correlate with that seen for IL2 mRNA, suggesting that both promoters are responsive to a similar signaling pathway. Induction of META did not result in a corresponding increase in transcription from the conventional promoter in the 5' LTR. A rapid increase in transcription from this promoter is associated with glucocorticoid stimulation in mammary tissue during lactation and is dependent on hormone-receptor binding to the GRE within the U3 region. Proviral transcription is also upregulated in normal B cells and several B cell lines in response to dexamethasone or lipopolysaccharide (LPS) and enhanced expression may correlate with B cell differentiation. (107, 108). As discussed in Chapter 1, a high level of constitutive expression of endogenous MMTV provirus has been observed in

Figure 3-2 Activation-dependent accumulation of IL2 and META mRNAs in EL4.E1 cells

EL4.E1 HP2 cells were; untreated, treated with 15 ng/ml PMA and 1.5 μM ionomycin or PMA, ionomycin and 100 ng/ml of CsA for varying times prior to the isolation of total RNA. Northern analysis was performed as described in Chapter 2 using 15 μg of RNA per tract. The blot was probed consecutively with ^{32}P end-labeled oligonucleotides MIL2.A01 and Mtv.A08 (Table 2-1) and final washes were done at 45°C in 6XSSC, 0.1% SDS. As a control the blot was also probed with cDNA corresponding to a ribosomal protein from the L32 gene family (44).


EL4.E1 cells- Time post-induction (hours)
1.75
3.25
4.5
6.0
6.0+CsA
7.5
9.0
9.5
11.25
15.25
23.5
23.5+CsA
Untreated
MMTV mRNA
Full length
28 S
env mRNA
18 S
META transcript
IL2 mRNA
Ribosomal protein mRNA

non-mammary tumor cell lines. These tumor lines contain amplified copies of the MMTV provirus and the LTRs are altered by deletions or deletion/insertions within the U3 region (51, 155, 156, 209). Loss of transcriptional control could be due to removal or alteration of the GRE elements and/or negative regulatory elements associated with tissue specificity of expression. Deletions within the LTR may contribute to enhanced expression of MMTV proviral DNA in certain T lymphomas. In EL4.E1 cells, the META element and the LTR promoter appear to be regulated independently. Further experimentation is required to determine if this is indeed the case in additional T leukemic cells in which MMTV is amplified and contains alterations or deletions within the conventional LTR promoter.

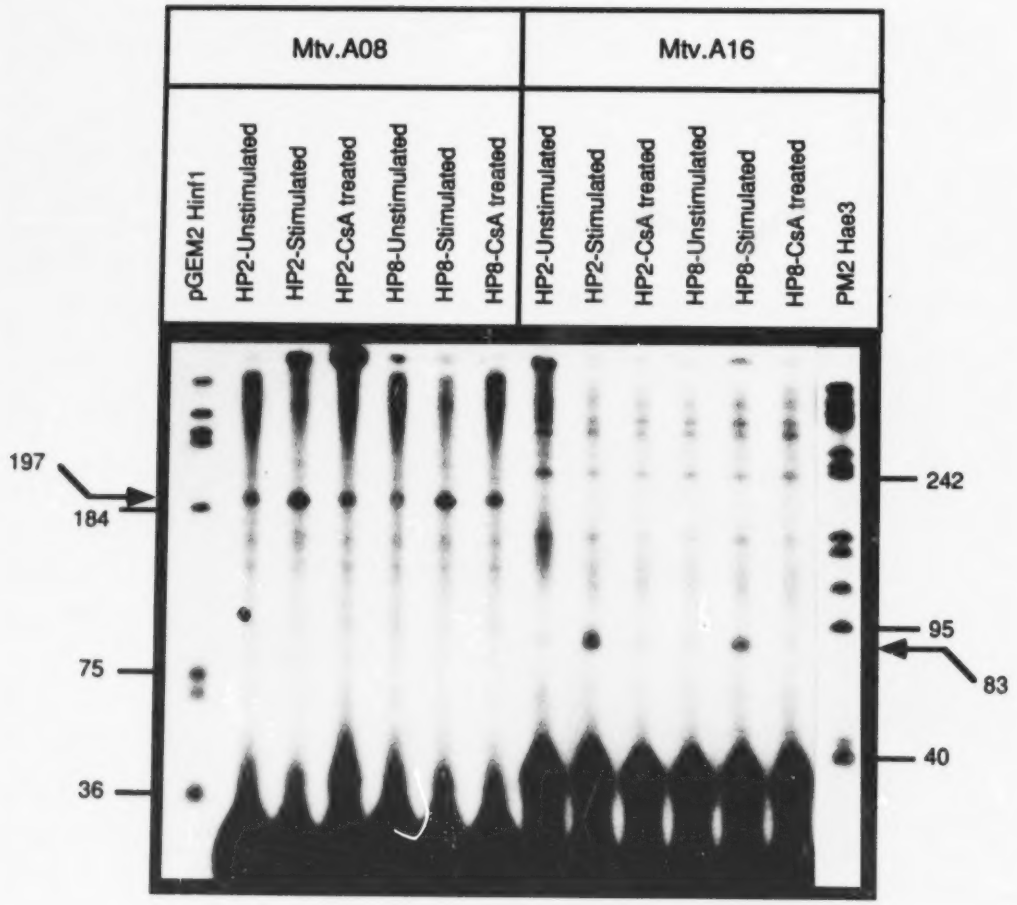
META transcription initiates within the MMTV *env* gene

Sequence analysis of cDNA clones, corresponding to META transcripts, indicated that the first nucleotide was at or near the A residue 7247 (51). To confirm the start site for the META transcript and to show that this segment of the *env* gene is a promoter in intact cells, MMTV RNA from induced EL4.E1 cells was examined by the primer extension technique. Total cellular RNA was isolated from unstimulated EL4.E1 HP2 and HP8 cells, from cells treated for 8 hr with 15 ng/ml PMA, or from cells treated with PMA and 100 ng/ml CsA. The oligonucleotide probe Mtv.A08, which has its 5' end at nucleotide 8611, was designed to detect processed transcripts, as it is positioned to the right of the intron (refer to Figure 2-1). The expected sizes of products using this primer were 204 bp for spliced mRNA and 1364 for non-spliced transcripts. A second oligonucleotide, Mtv.A16, primes to the 5' side of the intron, at position 7328 and therefore would not distinguish between spliced and non-spliced META transcripts. The expected product size was 86 bp. The results of the primer extension analysis are shown in Figure 3-3. The major product detected with primer Mtv.A08 was approximately 197 nt in length, while the Mtv.A16 product was 83 nt long. The predicted starting points of the mRNA are 7253 and 7250, respectively which correlates with META start site previously predicted (51). The increase induced by PMA was suppressed by CsA, corresponding to the pattern found using Northern analysis.

Results using primer Mtv.A08 suggest that unstimulated EL4.E1 cells contain RNA which yields products similar in size to that predicted for

Figure 3-3 Determination of META transcriptional start site in PMA-treated EL4.E1 cells using the primer extension assay

Total cellular RNA was isolated from two EL4.E1 clones, HP2 and HP8, which were either untreated, or treated with 15 ng/ml PMA in the presence or absence of 100 ng/ml CsA for 8 hr. Ten μg of RNA was used for each primer extension reaction. Primer Mtv.A08 begins at position 8611 and Mtv.A16 at position 7328 of the MMTV sequence (Table 2-1). The positions of marker PM2-Hae III or pGEM2-Hinf I DNA fragments are shown. Arrows indicate the predominant primer extension products and their lengths in nucleotides, as determined by the results of three experiments.


Mtv.A08
Mtv.A16
pGEM2 Hinf1
HP2-Unstimulated
HP2-Stimulated
HP2-CsA treated
HP8-Unstimulated
HP8-Stimulated
HP8-CsA treated
HP2-Unstimulated
HP2-Stimulated
HP2-CsA treated
HP8-Unstimulated
HP8-Stimulated
HP8-CsA treated
PM2 Hae3
197
184
75
36
242
95
83
40

processed META transcripts. It is unlikely that the majority of these products correspond to a mature META transcript because; (1) no META transcription is detectable in unstimulated EL4.E1 cells as assessed by Northern analysis (2) products of the appropriate size were obtained using the oligonucleotide primer Mtv.A16 and these products were present at low, or were undetectable, in uninduced cells. The larger products not resolved on this gel may represent non-spliced META transcripts or abortive reverse-transcribed cDNA corresponding to full-length or env mRNA.

In summary, an MMTV transcript does initiate within the *env* gene of MMTV provirus of EL4.E1 cells, and its expression is controlled by the same signals which induce or suppress cytokine transcription in these cells. These results raise the question of whether this transcription is activated by elements within the MMTV *env* gene (META), as distinct from more distal regulatory elements such as those in the LTR. The ideal experimental system would allow examination of META-driven transcription in the context of additional MMTV genomic sequences such as the LTR. Therefore we were interested in characterizing META with respect to cell type specificity of expression, responsiveness to activation of T cells and treatment by CsA, and whether META in isolation could account for the pattern of META expression seen in EL4.E1 cells.

Expression of a proteoglycan core protein, serglycin, in 12.1.19 cells

To study the transcriptional activating properties of the META region further (Chapter 4), it was desirable to use additional T cell lines. The T hybridoma cell line, 12.1.19 was provided by Dr. B. Singh, Department of Immunology, University of Alberta. This line is a subclone of the A1.1 cell line generated by Fotedar and coworkers (59) and secretes IL2 in response to presentation of its nominal antigen-poly 18 (poly [Glu-Tyr-Lys-{Glu-Try-Ala}$_5$) by IA^d-restricted antigen presenting cells, or by treatment with anti-CD3ε, or Con A. It thus resembles normal T cells in its activation requirements. Activation of IL2 production coincides with initiation of programmed cell death, or apoptosis, and both events are blocked by CsA (179).

As part of a more general analysis of transcription in activated T cells, we examined the expression of a proteoglycan core protein (PGCP). It was shown by this laboratory that EL4.E1 cells treated with PMA transcribed a PGCP serglycin and that induction of expression was not blocked by CsA.

The cloned cDNA was designated clone 154 (49). Experiments were done to determine if mRNA for serglycin or a closely related PGCP was also induced in the T cell hybridoma, 12.1.19.

Proteoglycans consist of sulfated polysaccharide chains, glycosaminoglycans (GAG) covalently linked to protein. The GAG chains are composed of repeating disaccharide units and exist in three main forms; (1) heparin sulfate and heparin, (2) chondroitin sulfate, (3) dermatan sulfate and keratin sulfate. These proteins are abundant in bone, cartilage and interstitial tissue, where they play a structural role in cell-cell and cell-matrix interaction. They are also important constituents of plasma and basement membranes of epithelial, endothelial and neuronal cells (reviewed in 85).

It has become increasingly clear that proteoglycans also have an important role as modulators of growth factor activity (reviewed in 126, 167). Specific proteoglycans with the capacity to bind polypeptide growth factors may act as accessory molecules for presentation of the growth hormone to its receptor or as a cell-surface reservoir. Binding of growth factors such a IL3 and GM-CSF by heparin and heparin sulfate associated with marrow stromal cells may facilitate their presentation to hemopoietic cells (164). Association with free heparin or a heparin sulfate proteoglycan, most likely syndecan, is required for binding of basic fibroblast growth factor to its high affinity receptor (215). Platelet factor 4, present in the α–granules of platelets is associated with the chondroitin sulfate form of serglycin proteoglycan (149). In the examples discussed, the growth factors recognize the GAG component of the proteoglycan but there is a least one example where binding is mediated by the core protein. Transforming growth factor-β (TGF-β) has been shown to bind via its core protein to the proteoglycans, betaglycan (3, 25) and decorin (213). Association with membrane-anchored betaglycan is not required for binding of TGF-β to its signaling receptors but may act as a cell surface reservoir to prevent rapid clearance of TGF-β upon release or provide a mechanism for regulating the amount of free cytokine. Decorin, associated with the cell matrix, has been shown to reversibly bind TGF-β and neutralize its activity. Synthesis of decorin is stimulated by TGF-β in various cells and therefore one function of this proteoglycan may be to negatively regulate TGF-β activity (213).

Serglycin proteoglycans are produced by various bone marrow derived cells. They are present within the secretory granules of mast cells,

basophils, NK cells and platelets and are released in response to certain stimuli (reviewed in 111). One function of serglycin proteoglycans may be to stabilize and concentrate proteins present within the secretory granules. They appear to complex with basic proteins within the acidic granules and dissociate from them following secretion from the cell. Serglycin CP was first cloned and sequenced from a rat yolk sac tumor (17). Closely related sequences have been obtained from a variety of cell types including mouse mast cells (6) and the human HL-60 promyelocytic leukemia cell line (5). Serglycin is so named for a long repeating sequence of serine and glycines, the serines being potential sites for GAG synthesis. Although chondroitin sulfate is the predominant GAG linked to serglycin CP, chondroitin 4-sulfate or heparin may also be utilized (111). Serglycin proteoglycans are relatively protease resistant, likely due to dense packing of the GAG on the core proteins.

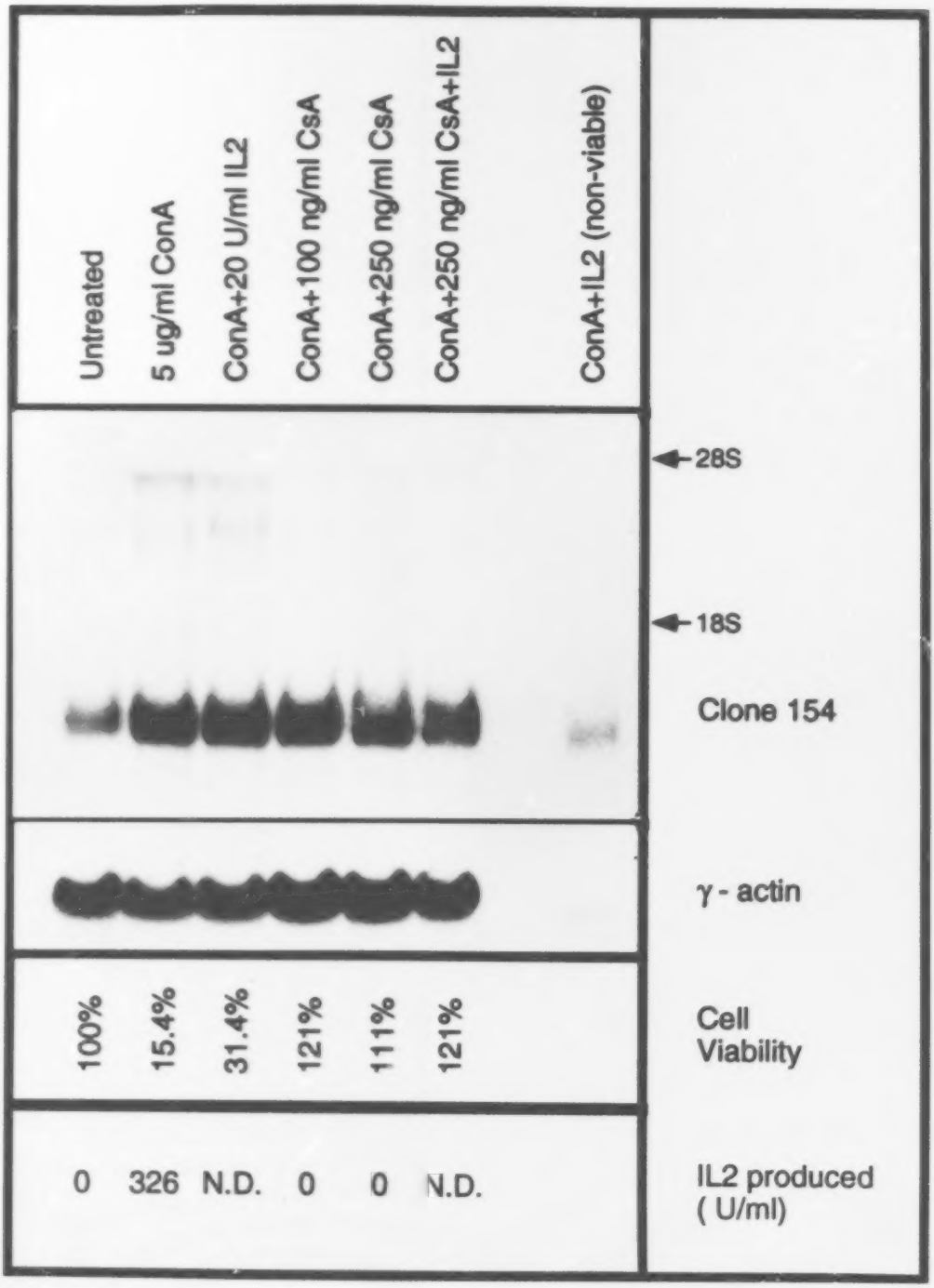
It is not known whether T lymphocytes produce serglycin proteoglycans. It has been shown that human T lymphocytes secrete a protease-resistant chondroitin sulfate proteoglycan in response to Con A (29, 30). The core protein could potentially be serglycin or a closely related protein. In this study we demonstrate that serglycin CP mRNA was induced in the T hybridoma cell line 12.1.19 by Con A and show that the increase in expression was not blocked by the immunosuppressive drug CsA.

12.1.19 cells at a density of $3.3x10^5$/ml were separated into the following groups; unstimulated, 5 µg/ml of Con A, Con A + 20U/ml IL2, Con A+100 ng/ml CsA, Con A + 250 ng/ml CsA and Con A + 250 ng/ml CsA + IL2. The cells were treated with the indicated agents for 20 hr and non-viable cells were removed by centrifugation through a Ficoll density gradient prior to the isolation of total RNA. Due to the predominance of dead cells in groups where 12.1.19 had been activated by Con A, the non-viable cells were also washed and RNA isolated. An aliquot of media was retained for determination of IL2 levels. Northern analysis was performed using 15 µg of RNA per track and probing with ^{32}P-labeled serglycin CP cDNA (Figure 3-4).

There was a significant level of serglycin CP mRNA transcription in untreated cells, which increased upon treatment with Con A. CsA at concentrations up to 250 ng/ml did not alter this enhanced expression whereas IL2 production was completely blocked by 100 ng/ml of CsA. Upon activation, 12.1.19 cells undergo apoptosis, resulting in a significant decrease in number

Figure 3-4 Increased expression of a proteoglycan core protein, serglycin, in an activated T-cell hybridoma

12.1.19 cells at a density of $3.3x10^5$/ml were separated into the following treatment groups; untreated, 5 µg/ml of Con A, 5 µg/ml Con A + 20 U/ml IL2, 5 µg/ml Con A + 100 ng/ml CsA, 5 µg/ml Con A + 250 ng/ml CsA and 5 µg/ml Con A + 250 ng/ml CsA + 20 U/ml IL2. The cells were treated with the indicated agents for 20 hr and non-viable cells were removed by centrifugation through a Ficoll density gradient prior to the isolation of total RNA. RNA was also isolated from non-viable cells (those present below the density gradient) from cells treated with 5 µg/ml Con A + 20 U/ml IL2. IL2 levels in the supernatant were measured using the MTT assay as described in Chapter 2 and cell viability was assessed using eosin dye exclusion. For Northern analysis, 15 µg of RNA was loaded per track and the blot was probed with serglycin CP cDNA (49) or human gamma-actin cDNA. The Northern blot was hybridized overnight at 42°C and washed at a final stringency with 0.2XSSC, 0.1% SDS at 60°C in both cases.


Untreated
5 ug/ml ConA
ConA+20 U/ml IL2
ConA+100 ng/ml CsA
ConA+250 ng/ml CsA
ConA+250 ng/ml CsA+IL2
ConA+IL2 (non-viable)
28S
18S
Clone 154
γ - actin
100%
15.4%
31.4%
121%
111%
121%
Cell Viability
0
326
N.D.
0
0
N.D.
IL2 produced (U/ml)

of viable cells recovered. Only 15% of Con A treated cells were recovered when compared to the cell number obtained in the untreated control sample. However, the Con A-induced increase in serglycin CP expression was essentially the same as samples in which both IL2 production and apoptosis were suppressed by CsA. The final track represents RNA from non-viable cells treated with Con A and 20U/ml of IL2. The relative levels of serglycin CP RNA and actin mRNA could not be determined due to low recovery of intact RNA, likely the result of RNA degradation associated with initiation of programmed cell death.

In addition to being induced in the cell lines, EL4.E1 and 12.1.19, serglycin mRNA also increased during the generation of an allogeneic MLR. It was also detected in the bone-marrow derived cells including P815 (mastocytoma), MTL2.8.2 and CTLL (mouse CTL), RI (B cell line), S194 (T cell) but was not detectable in two fibroblast cell lines, L cells and 3T3 (49). Serglycin proteoglycans could potentially be contained within cytoplasmic granules of the CTL cell lines CTLL and MTL2.8.2 and in cytotoxic cells generated during the MLR. Cytoplasmic granules of CTL contain chondroitin sulfate proteoglycans associated with the cytolytic effector molecules perforin and granzymes.

The potential function of serglycin proteoglycans in the T helper cell lines is unclear. It would be interesting to determine if any form of this proteoglycan has the ability to bind cytokines. It is conceivable that proteoglycans elaborated upon activation of a T helper cell, may reversibly bind cytokines produced by that or adjacent cells. The binding may extend the biological half-life of the cytokine and provide a mechanism for modulating or sustaining an immunological or proliferative response within a specific tissue microenvironment.

It has been hypothesized that the diversity of function of proteoglycans is related more closely to the structural variation of the GAG side chains than the type of core protein present (85, 111). The GAG can vary with respect to number of chains, length of the chains and their pattern of sulfation Although the serglycin CP is expressed, characterization of the function of the serglycin proteoglycan in the various cell types requires further analysis of the structure of GAG chains.

CHAPTER FOUR. IDENTIFICATION OF AN INDUCIBLE, CsA-SENSITIVE TRANSCRIPTIONAL ACTIVATOR IN THE MMTV ENVELOPE GENE[1]

Introduction

It has been demonstrated that when certain clonal isolates of the mouse EL4 thymoma cell line are stimulated by PMA, a high level of transcription is initiated from the MMTV provirus (51, 115, Chapter 3). In contrast to the normal mode of transcription in MMTV and other retroviruses, which starts within the right-hand part of the 5'-LTR, most of the PMA-induced transcription initiates from within the envelope gene, which lies just upstream of the 3'-LTR of MMTV. After processing the PMA-inducible transcript comprises primarily the 3' LTR. Induction of this transcription parallels that of the IL2 gene in EL4.E1 cells, in that expression is activated by the same inductive signal, and inhibited by the immunosuppressive agent CsA (177, Chapter 3). These observations suggest that there is a novel promoter/enhancer, within the MMTV *env* gene which is activated in response to signals which induce cytokine transcription in T helper lymphocytes. This promoter/enhancer generates a transcript which contains the ORF of the 3' LTR. Recent work indicates that this ORF encodes a protein responsible for the Mls phenotype (2, 61, 125, 153).

In this study we isolated MMTV proviral *env* gene segments, from EL4.E1 cells, a T cell hybridoma, and from BALB/c spleen cells. These elements (called META for MMTV *env* transcriptional activator) when linked to the reporter gene Chloramphenicol acetyl transferase (CAT) and introduced into several T helper cell lines, displayed properties similar to those shown for the endogenous META element present in EL4.E1 cells. Activation of the transfected cells with stimuli which give rise to IL2 production resulted in the induction of CAT activity. The induced activity of most of the META elements tested was sensitive to CsA. Only a very low

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[1]Some of the data presented in this chapter are included in a manuscript titled "An activation-dependent, T-lymphocyte specific transcriptional activator in the Mouse Mammary Tumor Virus env gene" published in Molecular and Cellular Biology 12:3262-3272. 1992. Written permission has been obtained from the journal to use these data in this thesis.

background level of activity was obtained in the absence of T cell activation or in non-T helper cell lines. When a 412 bp portion of the META element was linked to a heterologous promoter, it functioned as an inducible, orientation-independent, CsA sensitive enhancer in T helper cells.

Isolation of MMTV proviral sequences

Virtually all inbred mouse strains contain MMTV provirus integrated into the host genome at specific chromosomal locations. These loci segregate independently and can be used as genetic strain markers. EL4.E1 cells, in addition to having the MMTV proviral loci *Mtv-8*, *Mtv-9* and *Mtv-17* associated with the C57Bl/6 background, have amplified provirus copies which contain within their LTR a 494 bp deletion (51, 115). It is these deletion versions which are the template for PMA-induced transcription (51). It is unknown if all, or only a portion of the amplified provirus in EL4.E1 cells are competent for transcription of META RNA and whether cis-acting elements other than those present in the *env* gene contribute to the pattern of META expression documented; namely inducible, CsA-sensitive expression paralleling that of the IL2 gene.

To isolate fragments of META and study its properties, a representative sampling of the right hand portion of MMTV proviral sequences from EL4.E1 cells, PCR products extending between nucleotides 6750 and 9901 were generated. This 2.7 kb segment includes about 500 nucleotides upstream of the META transcript start site and extends 3' through the remainder of the *env* gene and the deletion-containing 3' LTR (Figure 2-1). As detailed in Chapter 2, the PCR products were ligated into the multiple cloning site (MCS) of pGEM3Z and 20 individual clones were randomly selected for further study. In principle, an individual MMTV clone could represent a gene capable of expressing the META transcript, as it may contain the *env* promoter/enhancer, intronic splice-donor and splice-acceptor sites and a polyadenylation signal within the 3'LTR. The plasmid pGEM3Z does not contain any defined eukaryotic transcriptional control elements in proximity of the MCS. It was hypothesized that a portion of the isolated EL4.E1-derived clones in pGEM3Z would be capable of expressing the META transcript in transient transfection assays.

Results of preliminary transfection experiments

Initially we intended to study META expression directly, by transfecting the recombinant plasmids into a suitable T-cell line by the DEAE-dextran procedure and following activation, isolate RNA for analysis. Because EL4.E1 cells have a high constitutive level of MMTV RNA transcription and express the META transcript upon activation, they were unsuitable as recipient cells if the product to be assayed was RNA. The T hybridoma 12.1.19 was therefore chosen for use, because it is more representative of a normal T helper and produces IL2 in response to several stimuli, including antigen. Crucial for the proposed experiments, northern blot analysis and primer extension did not detect any MMTV mRNA including the short, META-controlled transcript found in activated EL4.E1 cells from either untreated or Con A treated 12.1.19 cells (results not shown). For comparison, the (6750/9901) segment was also generated by PCR from 12.1.19 genomic DNA. The expected 3.2 kb products were obtained and cloned into pGEM3Z.

Two clones derived from 12.1.19 cells and the 20 clones from EL4.E1 cells were selected for use in transient expression assays. Recombinant pGEM3Z[MMTV(6750/9901)] plasmids were purified by CsCl density gradient centrifugation and introduced into 12.1.19 cells using the DEAE-dextran procedure. Following a 15 to 18 hr post-transfection incubation, the cells were treated with 5 µg/ml Con A and harvested 5, 9 or 18 hr later. The control cells were treated identically with the exception of Con A stimulation. Total RNA was isolated by the guanidine thiocyanate-CsCl density gradient procedure. In several experiments the RNA was further purified by phenol-chloroform extraction prior to precipitation with sodium acetate/ethanol. RNA was also isolated from PMA-treated non-transfected EL4.E1 cells. Several techniques were employed in an attempt to identify RNA originating from the transfected plasmids.

Primer extension analysis was done using up to 150 µg of total RNA from transfected cells and either Mtv.A08 or Mtv.A16 ^{32}P-labeled oligonucleotide primers (Table 2-1). These primers were utilized successfully to detect META transcripts with 10 µg of RNA from PMA-treated EL4.E1 cells (Figure 3-3). However, primer extension products were undetectable even using a 15 fold higher amount of RNA from transfected 12.1.19 cells.

The RNase protection assay was also employed, as internal labeling of the synthetic RNA probe allows greater sensitivity of detection than attain-

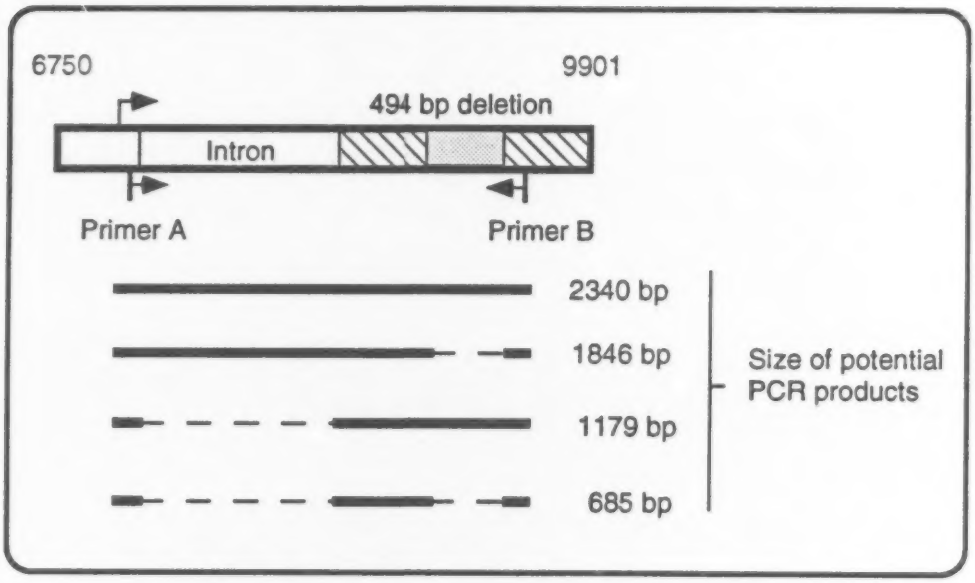
able with the primer extension technique using end-labeled primers. The vector used to generate the antisense RNA probe was constructed using a portion of the cDNA of clone 14. Clone 14 is a prototypic cDNA obtained from the subtracted EL4.E1 library made by Dr. J. Elliot (51). Using 5 µg of RNA from PMA-treated EL4.E1 cells, a band of the appropriate size was detected (results not shown) and a very weak signal was present in several samples containing 150 µg RNA from transfected Con A treated 12.1.19 cells. Neither the primer extension technique nor the RNase protection assay yielded conclusive data indicating which (if any) of the transfected plasmids were capable of expressing MMTV RNA. It is possible that neither technique was sensitive enough to detect META transcripts, if indeed, they were being produced in the transfected cells.

The final procedure used involved reverse transcription of RNA isolated from transfected 12.1.19 cells followed by PCR amplification (cDNA-PCR). Five to 10 µg of test RNA was reverse-transcribed using an oligonucleotide primer which was complementary to the 3' LTR downstream of the 494 bp deletion which occurs in EL4.E1 MMTV DNA. Ten percent of this reaction was amplified by PCR using the same downstream primer and a sense primer corresponding to nucleotides located to the right of the META intronic splice site within the *env* gene. The primers selected would potentially yield various sized products, depending on whether the cDNA amplified corresponded to MMTV RNA which had been spliced and/or was shorter in size due to the 494 bp-LTR deletion. A schematic and explanation of the potential PCR products are shown in Figure 4-1. Although several experiments yielded tantalizing results, the control samples were not always reproducible therefore rendering the results uninterpretable.

In retrospect, there are several technical reasons why the cDNA-PCR approach was not successful. One involves control experiments in which reverse transcriptase was omitted. Using RNA from non-transfected PMA-treated EL4.E1 cells, PCR products corresponding to spliced and non-spliced RNA (685 bp and 1846 bp respectively) were obtained. On several occasions products of a similar size were present in control samples. Because I did not obtain reproducible data with the control samples, I could not exclude the possibility that RNA isolated from transfected 12.1.19 cells contained contaminating plasmid even with the use of DNase I treatment. Although we considered the possibility at the time, it has since been suggested that Taq

Figure 4-1 Sizes of potential PCR products obtainable using RNA from transfected 12.1.19 cells

The boxed area represents the (6750/9901) MMTV segment isolated from either 12.1.19 or EL4.E1 genomic DNA. The hatched area represents the 3' LTR which may also contain, in the case of the EL4.E1 clones, a 494 bp deletion (stippled area). The approximate positioning of the 1.2 kb intronic sequence of the META transcript is shown. Primer A (sense) is positioned upstream of the intron boundary and downstream of the putative transcriptional start site for the META transcript. The antisense primer B corresponds to sequence situated to the right of the 494 bp deletion. The solid lines indicate DNA which may be in the PCR products and the broken lines represent sequence which may be absent due to presence of the 494 bp deletion and/or removal of the intron.


6750
9901
494 bp deletion
Intron
Primer A
Primer B
2340 bp
1846 bp
1179 bp
685 bp
Size of potential
PCR products

polymerase itself is capable of reverse transcriptase activity thereby explaining the anomalous results. The test system itself was technically difficult in that I could not predict if, or when, transcription from the transfected plasmids was peaking and induction of apoptosis by treatment of the 12.1.19 with Con A made recovery of non-degraded RNA challenging.

In summary, the right portion of the MMTV provirus containing the putative META element, the remainder of the *env* gene and the 3' LTR was isolated from EL4.E1 genomic DNA. Attempts were made to assess the capability of this DNA to direct transcription in transient transfection assays in a T hybridoma cell line activated by Con A. Primer extension, RNase protection and reverse transcription followed by cDNA-PCR protocols were used. However this approach yielded no conclusive data demonstrating that transcription was induced from the META element and was abandoned in favor of a more conventional approach, that of attaching the putative transcriptional activator elements (META) to the reporter gene, Chloramphenicol acetyl transferase (CAT).

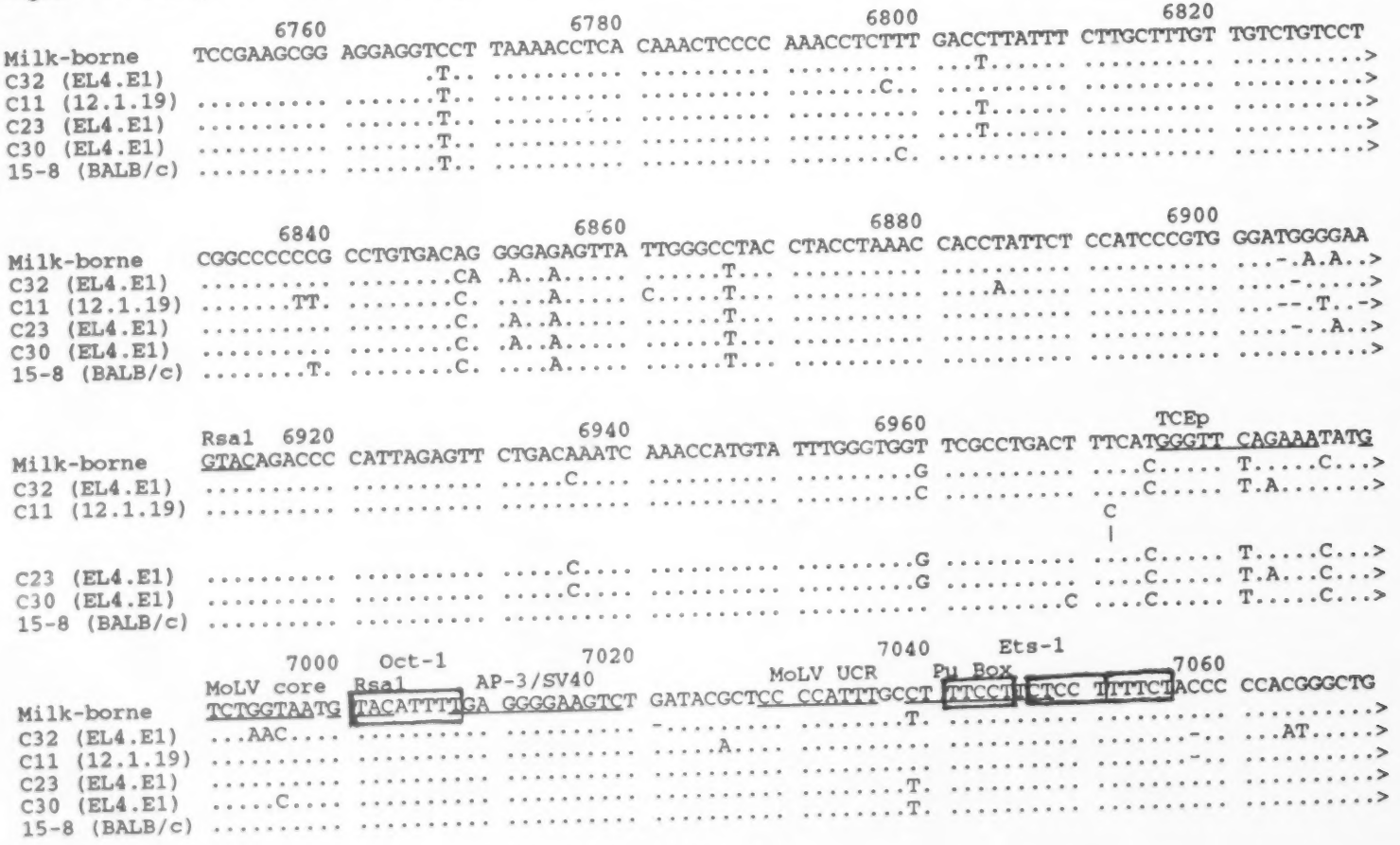
Isolation of the META element and cloning into CAT expression plasmids

As described in Chapter 2, sequences presumed to contain the putative META element were excised from the pGEM3Z [MMTV(6750/9901)] clones and inserted into an expression vector upstream of the coding region for CAT (Figure 2-2). The cloned sequences C30, C23 and C32 were derived from EL4.E1 DNA, clone C11 from 12.1.19 genomic DNA, and clone 15-8 was generated from BALB/c spleen cells. A 505 bp region of the *env* gene which includes the TATA box and start site of META transcription from each of the EL4.E1 and 12.1.19 isolates was cloned into pGEM2(cat), as were the corresponding LTR sequences. The homologous region (6750/7328) of BALB/c spleen cell MMTV was also inserted into the CAT expression plasmids. Sequence analysis confirmed that the EL4.E1 DNA clones selected contained the 494 bp deletion of the amplified provirus forms. Unexpectedly, the 3'-LTR of the 12.1.19 clone contained a substitution, in which the region (9462/9552) was replaced by 107 base pairs of unknown origin. This substitution alters the distal GRE element. There was no evidence, based on Southern blot analysis (data not shown) that 12.1.19 cells contain amplified MMTV provirus, and MMTV RNA does not appear to be expressed constitutively or in response to activation.

Figure 4-2 Sequence comparison between milk-borne MMTV and C32(6750/7255), C11(6750/7255), C23(6750/7255), C30(6750/7255) and 15-8(6750/7328).

The milk-borne sequence is numbered as in Moore et al. (138). Sequence identity between the META isolates and the milk-borne proviral sequence is shown by a dot, and gaps by a dash. The 505 bp META fragments from C30 and C11 were terminated at the Dpn I site at 7255, while the 15-8 sequence was terminated at 7328. Transcription of the PMA-induced RNA in EL4.E1 cells initiates at 7247 (see text), 30 nucleotides downstream from the putative TATA box centered at 7217. There is a CCAAT sequence at 7196 and a TATA-like sequence at 7122. Additional sequences which are similar to known regulatory protein binding motifs are indicated and described in Table 4-4.

Figure 4-2 Comparison of META sequences to MMTV milk-borne sequence


6760 6780 6800 6820
Milk-borne TCCGAAGCGG AGGAGGTCCT TAAAACCTCA CAAACTCCCC AAACCTCTTT GACCTTATTT CTTGCTTTGT TGTCTGTCCT
C32 (EL4.E1) .T.. ...T.....
C11 (12.1.19) .....T.. .....C..
C23 (EL4.E1) .....T.. ...T.....
C30 (EL4.E1) .....T.. .....C.
15-8 (BALB/c) .....T..
6840 6860 6880 6900
Milk-borne CGGCCCCCCG CCTGTGACAG GGGAGAGTTA TTGGGCCTAC CTACCTAAAC CACCTATTCT CCATCCCGTG GGATGGGGAA
C32 (EL4.E1) .....CA .A..A..... .....T... ....A..... ...-.A.A..>
C11 (12.1.19) .....TT. .....C. ....A..... C.....T... ....A..... ....-.....>
C23 (EL4.E1) .....C. .A..A..... .....T... ...--.T..>
C30 (EL4.E1) .....C. .A..A..... .....T... ....-..A..>
15-8 (BALB/c) .....T. .....C. ....A..... .....T... >
Rsa1 6920 6940 6960 TCEp
Milk-borne GTACAGACCC CATTAGAGTT CTGACAAATC AAACCATGTA TTTGGGTGGT TCGCCTGACT TTCATGGGTT CAGAAATATG
C32 (EL4.E1) .....C.... .....G..... ....C..... T.....C...>
C11 (12.1.19) .....C..... ....C..... T.A.....>
C
C23 (EL4.E1) .....C.... .....G..... ....C..... T.....C...>
C30 (EL4.E1) .....C.... .....G..... ....C..... T.A...C...>
15-8 (BALB/c) .....C ....C..... T.....C...>
7000 Oct-1 7020 7040 Ets-1
MoLV core Rsa1 AP-3/SV40 MoLV UCR Pu Box 7060
Milk-borne TCTGGTAATG TACATTTTGA GGGGAAGTCT GATACGCTCC CCATTTGCCT TTCCTTCTCC TTTTCTACCC CCACGGGCTG
C32 (EL4.E1) ...AAC.... .....T. .....-.. ...AT.....>
C11 (12.1.19) .....A..... .....-.. >
C23 (EL4.E1) .....T. >
C30 (EL4.E1) .....C.... .....T. >
15-8 (BALB/c) >
77

```
                      7080                                                  7120                                                  7140
Milk-borne     CTTTCAAGTA GACAAGCAAG TATTTCTTTC TGATACACCC ACGGTTGATA ATAATAAACC TGGGGGAAAG GGTGATAAAA

C32 (EL4.E1)   .......... ..T....... .......... .A........ .......... .......... .......... ..........>
C11 (12.1.19)  T.....G... ..T....... .......... .......... .......... .......... ........G. ..........>
                 A       T
                 |       |
C23 (EL4.E1)   .......... ..A....... .......... .......... .......... .......... ....A..... ..........>
C30 (EL4.E1)   .......... ..T....... .......... .......... .......... .......... .......... A.........>
15-8 (BALB/c)  .......... ..T....... .......... .......... .......... .......... .......... ..........>

                      7160          HincII   7180                                 7200                                  7220  CD28RO
Milk-borne     GGCGTATGTG GGAACTTTGG TTGACTACCT TGGGGAACTC AGGGGCCAAT ACAAAACTGG TCCCTATAAA AAAGAAGTTG
C32 (EL4.E1)   .......... A......... ........T. .......... .......... .......... .......... ..........>
C11 (12.1.19)  .......... .......... .......... .......... G......... .......... .......... ..........>
C23 (EL4.E1)   .A........ .......... ........T. ..A....... .......... .......... .......... ..........>
C30 (EL4.E1)   .......... .A........ ........T. .A.A...... .......... .......... .......... ..........>
15-8 (BALB/c)  .......... .......... .......... .......... G......... .......... .......... ..........>

                      7240          |----> Dpn1                             7280                                  7300
Milk-borne     CCCCCCAAAT ATCCTCACTG CCAGATCGCC TTTAAGAAGG ACGCCTTCTG GGAGGGAGAC GAGTCTGCTC CTCCACGGTG
C32 (EL4.E1)   .......... .......... .....
C11 (12.1.19)  .......... .......... .....
C23 (EL4.E1)   .......... .......... .....
C30 (EL4.E1)   .......... ..-....... .....
15-8 (BALB/c)  .......... .......... .......... .......... .......... .......... ..................>


Milk-borne     GTTGCCTTGC GCCTTCCCTG
15-8 (BALB/c)  ..................
```

The sequences of the cloned *env* fragments of C11, C30, C23, C32 and 15-8 are compared to that of the milk-borne virus in Figure 4-2 and are discussed in a later section.

Transient expression of CAT constructs

To determine the transcription-activating function of the MMTV *env* region fragments, plasmid DNAs containing the various segments were used to transiently transfect EL4.E1 cells by the DEAE dextran procedure. As shown in Figure 4-3, the 505 bp env fragment derived from clone C30, extending from MMTV positions 6750 to 7255 and designated C30(6750/7255), and the comparable fragment from clone C11, activated CAT gene expression in transfected cells which were stimulated with PMA, but not in unstimulated cells. This transcriptional activity was largely blocked by CsA. C30 in the reverse orientation, designated C30(7255/6750) was inactive. The 3' LTR fragments from C30 and C11 did not support CAT expression in untreated or PMA-induced EL4.E1 cells even though they contain the conventional MMTV LTR promoter. C23(6750/7255) was also inactive in both untreated and PMA-treated EL4.E1 cells (data not shown). The control plasmid containing a Rous Sarcoma Virus (RSV) promoter/enhancer was highly active under all conditions, as expected.

META supports CAT activity only in activated T helper cell lines

The transcriptional activating properties of the META-CAT constructs were tested in several types of cells, with the results shown in Figure 4-4. C11(6750/7255) supported inducible CAT gene expression in Jurkat cells (human T leukemia cell line) and in the mouse T hybridoma 12.1.19. A second EL4.E1-derived META fragment, C32(6750/7255), showed the same pattern of activation in 12.1.19 cells. The characteristics of C11(6750/7255) induction in Jurkat cells were the same as those for induction of the IL2 gene in these cells, namely, Con A and PMA alone were weak inducers, but synergized to yield strong induction. CsA blocked the induction of CAT activity in both Jurkat and 12.1.19 cells, as has also been found for the IL2 gene (177).

C30(6750/7255) and C11(6750/7255) were inactive in HeLa cells either untreated or after treatment with Con A or PMA supporting the conclusion that the specificity of induction was not due solely to the agent used, but also depended on the cellular context. The RSV promoter/enhancer was highly

Figure 4-3 Induction of CAT activity in EL4.E1 cells by PMA and suppression by CsA

EL4.E1 cells were transfected with META-containing constructs from two different clones (C30 and C11), or the LTR fragments from the same clones, or C30 in the reverse orientation (7255/6750), all attached to a promoter-less CAT gene. C30 was derived from EL4.E1 cells, and C11 from 12.1.19 T hybridoma cells. The positive control is the pRSV(cat) plasmid (75). Cells were either unstimulated, or stimulated with 15 ng/mL PMA in the presence or absence of 100 ng/mL CsA for the final 15 hr of a 42 hr incubation period following transfection.

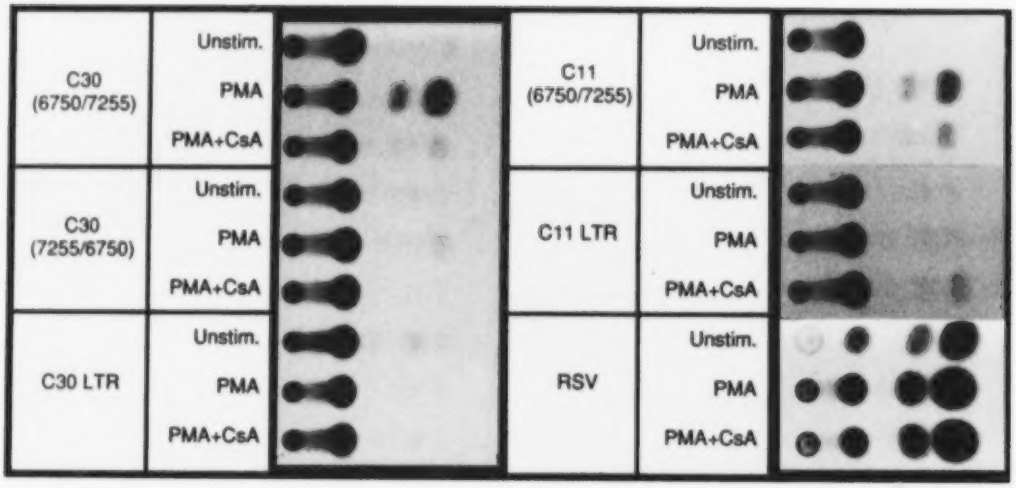
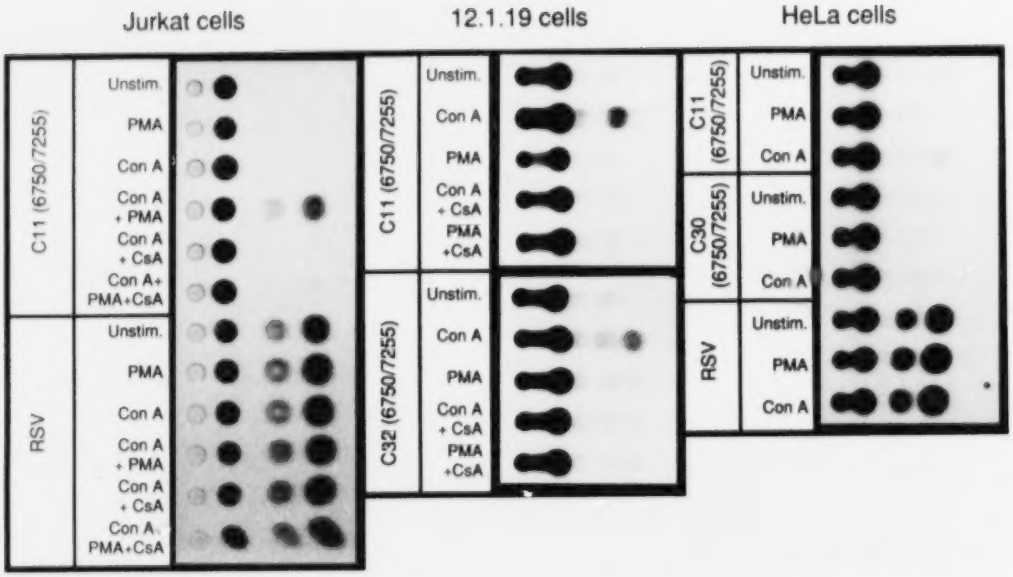

C30 (6750/7255)
Unstim.
PMA
PMA+CsA
C30 (7255/6750)
Unstim.
PMA
PMA+CsA
C30 LTR
Unstim.
PMA
PMA+CsA
C11 (6750/7255)
Unstim.
PMA
PMA+CsA
C11 LTR
Unstim.
PMA
PMA+CsA
RSV
Unstim.
PMA
PMA+CsA

Figure 4-4 Specificity of META function

Transfection with the indicated constructs was performed in Jurkat (human T leukemia), 12.1.19 (mouse T cell hybridoma) or HeLa (human epitheloid carcinoma) cells. Jurkat cells were treated with 10 ng/mL PMA and/or 30 mg/mL Con A; 12.1.19 and HeLa cells with 15 ng/mL PMA or 10 mg/mL Con A. CsA was 100 ng/mL. An equivalent amount of protein was assayed for each cell type, with the exception of 12.1.19 cells treated with Con A, where one-half the amount was used. C32 is another cloned MMTV sequence derived from EL4.E1 cells.


Jurkat cells
12.1.19 cells
HeLa cells
C11 (6750/7255)
RSV
Unstim.
PMA
Con A
Con A + PMA
Con A + CsA
Con A+ PMA+CsA
C11 (6750/7255)
C32 (6750/7255)
Unstim.
Con A
PMA
Con A + CsA
PMA +CsA
C11 (6750/7255)
C30 (6750/7255)
RSV
Unstim.
PMA
Con A

active in all of these cell lines, independent of cellular stimulation or suppression.

I was unable to detect an appreciable level of CAT activity in several other types of cell lines which were transfected with C11 or C30 constructs (Table 4-1). We detected a low level of activity (two-fold over untreated cells) in LBB 3.4.16 cells treated with PMA. This B cell line is known to express the MMTV-directed Mls^a antigen (144). No activity was detectable in S194 B cells, the P815 mastocytoma cell line, HeLa cells, or a cytotoxic T cell line.

Transfection experiments were also carried out on primary BALB/c spleen cells which had been incubated for 48 hr with Con A prior to transfection. No appreciable level of CAT activity was obtained with any of the MMTV containing constructs and only a low level of activity was seen with pRSV(cat). It is likely that the standard transfection protocol used was unsuitable for these cells.

The T hybridoma cell line 12.1.19 can be induced to synthesize IL2 by either Con A, or its nominal antigen, or anti-CD3ε antibody, but not by PMA. As shown in Figure 4-4, Con A induced CAT expression by C32(6750/7255) and C11(6750/7255) in 12.1.19 cells, whereas PMA alone had no effect. Figure 4-5 demonstrates that anti-CD3ε antibody can also induce CsA-sensitive transcriptional activation by the C30 and C11 constructs containing the 505 bp META fragment. The corresponding LTR fragments were non-functional under the same conditions. These results suggest that the (6750/7255) META fragments are capable of supporting transcription only in T helper cells, and then only following activation by the same stimuli which induce cytokine production in these cells.

Correlation of IL2 production and CAT activity in activated Jurkat T cells

I have demonstrated that the 505 bp segment isolated from EL4.E1 cells and 12.1.19 cells functions in a manner equivalent to that shown for the endogenous META element: expression was dependent on activation of the T helper cell and was CsA-suppressible. It has been shown that accumulation of the META transcript correlated with that of IL2 gene expression (Figure 3-2). Figure 4-6 demonstrates that upon activation of transfected Jurkat cells, accumulation of the CAT protein is parallel to the accumulation of IL2 activity. Jurkat cells transfected with pGEM2(cat)C30(6750/7255), were treated with ionomycin and PMA and harvested at the indicated intervals.

Table 4-1. Transcriptional activation by the 505 bp MMTV envelope segment in various cell lines

Cell Type	Unstimulated	Stimulated	(Agent)
T Helper Cell Lines			
EL4.E1	-	+	PMA
Jurkat	-	+	PMA + Con A
Jurkat	-	+	PMA+ionomycin
12.1.19 Hybridoma	-	+	Con A
12.1.19 Hybridoma	-	+	anti-CD3ε
B Cell Lines			
S194	-	-	PMA or Con A
LBB 3.4.16	-	-	LPS
LBB 3.4.16	-	+/-	PMA
Other			
P815, mastocytoma	-	-	PMA or Con A
HeLa, Endothelial carcinoma	-	-	PMA or Con A
MTL2.8.2, cytotoxic T cell	-	-	PMA or Con A

The indicated cell lines were transfected with C30(6750/7255) or C11(6750/7255) constructs in pGEM2(cat), and the indicated stimulating agents were added for the final 15 hr of culture. Results were graded on whether CAT activity obtained within any transfection group was greater than that obtained with mock transfected cells (no plasmid DNA present). A positive result represents a greater than 2-fold increase and less than a two-fold increase was considered insignificant. LPS - Lipopolysaccharide.)

Figure 4-5 Induction of META in a T-cell hybridoma by α-CD3ε
Cells of the T helper hybridoma line 12.1.19 were transfected with the C30 or C11-derived META or LTR constructs and treated with a monoclonal antibody to CD3ε (121a), in the presence or absence of 100 ng/mL CsA during the final 12 hr of incubation.

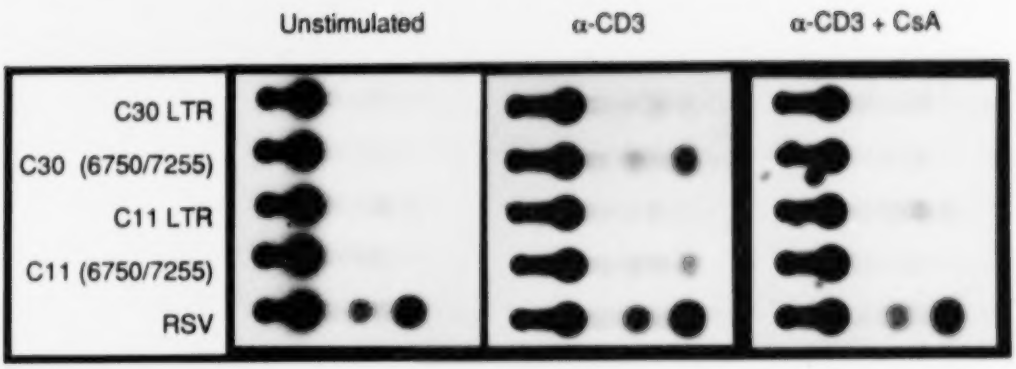
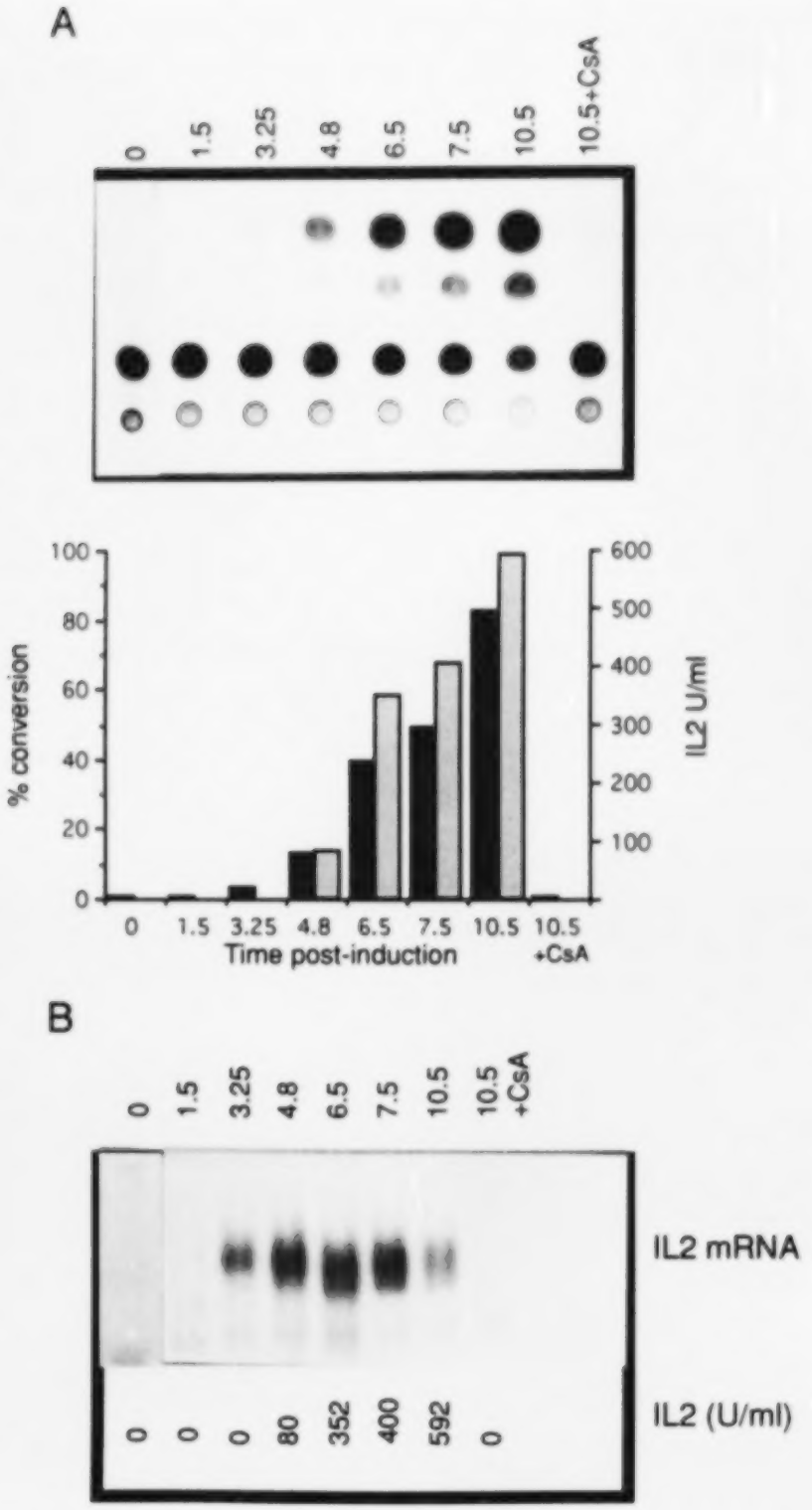

Unstimulated
α-CD3
α-CD3 + CsA
C30 LTR
C30 (6750/7255)
C11 LTR
C11 (6750/7255)
RSV

Figure 4-6 IL2 production correlates with META-driven CAT activity
Jurkat HP21 cells were transfected by the DEAE-dextran method with pGEM2(cat)C30(6750/7255) and recultured at $2x10^5$ cells per ml. Following an 32 hr incubation, cells were treated with 15 ng/ml PMA and 1.5 μM ionomycin, or PMA and ionomycin and 100 ng/ml CsA. The samples were harvested at the times indicated and the supernatant was retained for IL2 determination. A: Induction of CAT activity. The CAT assay was performed and quantitated as described in Chapter 2. Each assay point contained 250 μg of protein. CAT activity is expressed as percent conversion and is represented by the black bars. IL2 levels (U/ml) is shown as hatched bars. B: Total RNA was isolated by the guanidine thiocyanate/ CsCl gradient procedure and 20 mg per track was used for Northern analysis. The blot was hybridized overnight at 42°C with ^{32}P end-labeled oligonucleotide HIL2.A15 (Table 2-1), and washed with 6X SSC at 45°C. IL2 was quantitated using the MTT procedure.


A
0
1.5
3.25
4.8
6.5
7.5
10.5
10.5+CsA
100
80
60
40
20
0
% conversion
600
500
400
300
200
100
IL2 U/ml
0 1.5 3.25 4.8 6.5 7.5 10.5 10.5
Time post-induction
+CsA
B
0
1.5
3.25
4.8
6.5
7.5
10.5
10.5
+CsA
IL2 mRNA
0 0 0 80 352 400 592 0
IL2 (U/ml)

IL2 mRNA was detectable at 3.25 hr with biologically active protein detectable at 4.8 hr. By 10.5 hr, the IL2 mRNA levels had decreased dramatically although IL2 activity continued to accumulate. When compared to non-stimulated cells, there was a 10 fold increase of CAT activity detectable at 3.25 hours which accumulated to a 240 fold increase by 10.5 hr post-activation. Production of both IL2 and CAT proteins was sensitive to suppression by CsA. Although this experiment does not measure CAT mRNA directly, results suggest that it is induced shortly post-activation and there was appreciable expression of the CAT gene between 4 hr and 6 hr which is of relevance in selecting harvest times for certain types of analysis.

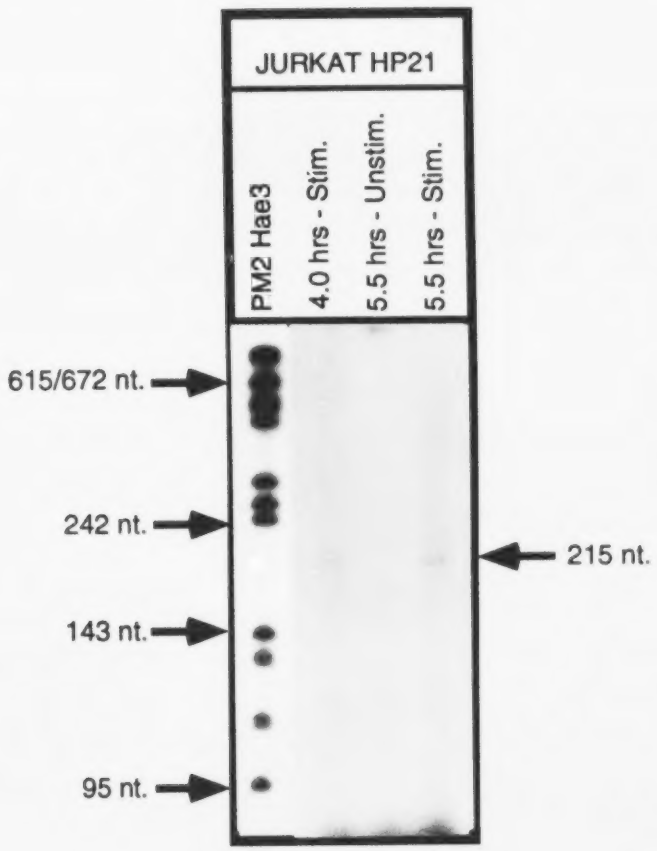
Localization of transcriptional start site of CAT transcripts in C30(6750/7255)

Although we demonstrated that the MMTV *env* gene contained a start site for the META transcript at or near position 7247 in intact EL4.E1 cells (51, Figure 3-3), we have not established whether the CAT mRNA encoded by the META-CAT constructs initiates at the corresponding position in transfection experiments. Northern blot analysis indicated that Jurkat cells do not express MMTV-related transcripts (data not shown). Unlike 12.1.19 cells, they do not undergo rapid apoptosis when treated with a combination of PMA and ionomycin, making them more suitable as a recipient cell line both for measurement of CAT activity and experiments where it is necessary to isolate RNA of good integrity. The level of CAT mRNA in transfected EL4.E1 and Jurkat cells appears to be very low, therefore primer extension could not be used to determine the start points of CAT-containing transcripts. Although RNase protection is more sensitive than primer extension, probes which contained MMTV antisense sequences hybridized to many endogenous RNAs and generated a high background of protected probe fragments when using RNA from EL4.E1 cells. This is not surprising, since there is constitutive expression of various MMTV RNAs (Figure 3-1 and 3-2).

RNase protection experiments were therefore done in Jurkat HP21 cells after transfection with pGEM2(cat)C30(6750/7255) (Figure 4-7). Total RNA was harvested 4 hr or 5.5 hr following activation with ionomycin and PMA. A protected fragment 236 nt in length was expected if the CAT transcripts initiated at position 7247 within the META element. A protected fragment was observed of 215 nt (average of two individual experiments)

Figure 4-7 Determination of start site in clone C30 META

Jurkat HP21 cells were transfected with plasmid pGEM2(cat)C30(6750/7255) and incubated for 21 hr prior to addition of 1.5 μM ionomycin and 15 ng/ml of PMA. The cultures were incubated for the indicated times and total RNA harvested. The RNA probe, 720 nt in length was generated using pCM2 (Figure 2-3). If the CAT mRNA initiated at position 7247 which correspond to the META transcript start site in EL4.E1 cells (Figure 4-1) an protected fragment 236 bp in length would be expected. RNase protection assay was done using 100 mg of total RNA and hybridization for 12 hr. The fragment indicated is calculated to be 215 nt in length based on the results of two experiments. The markers were derived by labeling PM2 DNA (^{32}P) which had been cut with Hae III.


JURKAT HP21
PM2 Hae3
4.0 hrs - Stim.
5.5 hrs - Unstim.
5.5 hrs - Stim.
615/672 nt.
242 nt.
143 nt.
95 nt.
215 nt.

suggesting that either the CAT mRNA was initiating at or just downstream of the expected start site, or from within the vector sequence which lies between the META element and the CAT coding sequence (Figure 2-2). Although we have never detected CAT activity over background levels using the pGEM2(cat) plasmid alone in transfection experiments, it is known that CAT expression vectors often contain cryptic promoters that give rise to enzymatic activity in the absence of inserted foreign DNA. Given the error inherent in the technique including the use of denatured DNA rather than labeled RNA as a sizing marker, these data suggest that META transcripts have either the anticipated start site or initiate just downstream from META.

Localization of transcription-activating sequences

The META element contains several motifs which are similar to known binding sites of trans-activating factors. Binding to one or more of these sites may be necessary to achieve inducible, tissue-specific expression. In order to localize the regulatory elements within META, a series of deletion fragments were ligated into the CAT reporter gene plasmids.

A series of fragments, generated using available restriction sites within the META region of the C30, C11 and 15-8 MMTV plasmids (Figures 2-1, 4-B) were cloned into the pGEM(cat) vectors. Jurkat cells were transfected and Con A and PMA, or ionomycin and PMA, were added for the final 12 hr of a 42 hr incubation period. Experiments using the different combination of inducing agents are shown in Tables 4-2A and 4-2B and are summarized schematically in Table 4-2C. The full length META fragments from isolates C30 and C11 and from clone 15-8, were included for comparison.

The sequence 15-8(6750/7328) induced CAT gene expression in stimulated Jurkat cells by over 100-fold when compared to untreated cells, but the induced expression was only moderately sensitive to CsA. Induction of transcription with the full-length constructs C11(6750/7255) and C30(6750/7255) was also strong, with both yielding greater than 100-fold enhancement, depending on experimental conditions. C30(6750/7255) showed the greatest sensitivity to CsA while C11(6750/7255) was inhibited by about 60-70%. When placed in the reverse orientation C30(7255/6750) was inactive but

surprisingly C11(7255/6750) had appreciable activity which was more than 85% suppressible by CsA.

Segments extending 3' from the Hinc II site, centered at 7172, although containing the TATAA sequence, were inactive in both orientations. This segment corresponded to C30 and C11 (7173/7255) and 15-8(7173/7328).

CAT activity of C30(7002/7255) was 18 to 20 fold over the level detectable in uninduced cells. The same fragment in the reverse-orientation yielded conflicting data. It was relatively inactive in the experiment presented in Table 4-2A whereas data given in experiment 2B suggested it functioned as an inducible non CsA sensitive promoter. The corresponding segments from the C11 clone were not appreciably active. This region contains a purine-rich segment which is almost identical to the core of the NF-AT binding sequence in the human and mouse IL2 genes (174, 178). These results suggest that although the region extending from 7002 to 7255 within the C30 clone derived from EL4.E1 cells is partially active in stimulated cells, and this activity is suppressible by CsA, sequences 5' to this region are required for full transcriptional activation. Several additional fragments, C11(6750/6912), C11(7002/7172), and C11(7172/7002) were inactive.

The segment (6750/7172) derived from all three clones, supported significant CAT expression only in activated Jurkat cells. The level of suppression of CAT activity by CsA was proportional to that obtained with the respective full-length segments. This segment does not contain a conventional TATAA motif, but the sequence TAATAA centered at position 7124 may function as a TATA box. The C30(7172/6750) construct was also active. The CAT mRNA could initiate downstream from this or other sites within the META deletion or from within the parental vector DNA. With available data, this point cannot be resolved.

To exclude the possibility that there is a bidirectional promoter present in the MMTV *env* gene, the Northern blot shown in Figure 3-1 was prehybridized for 4.5 hours at 58°C and hybridized with radiolabeled sense oligonucleotide Mtv.S02 for 3.5 hours at 58°C. The lack of specific binding of MTV.S02 suggested that there was no transcription of the non-coding MMTV DNA strand in the region surrounding position 6750.

As already described, it was difficult to determine the start sites of the CAT mRNA within the various plasmids used in deletion-analysis of the

Table 4-2A. Activation of CAT transcription by segments of the MMTV envelope gene

Promoter/enhancer	Untreated	Treated	Treated/CsA
pRSV	87	91 (1.0)	90 (1.0)
15-8 (6750/7328)	0.7	88 (122)	69 (96)
C11 (6750/7255)	0.3	81 (260)	25 (80)
C11 (7255/6750)	0.3	35 (116)	5.1 (17)
C11 (7002/7255)	0.2	0.4 (2)	0.4 (2)
C11 (7255/7002)	0.3	1.4 (5)	0.4 (1.5)
*C11 (7173/7255)	0.4	0.3 (1.0)	0.4 (1.0)
*C11 (7255/7173)	0.3	0.3 (1.0)	0.3 (1.0)
C30 (6750/7255)	0.3	42 (140)	1.1 (3.3)
C30 (7255/6750)	0.3	0.5 (2)	0.3 (1.0)
C30 (7002/7255)	0.5	9 (18)	0.9 (2)
C30 (7255/7002)	0.6	15 (25)	13 (21)
*C30 (7173/7255)	0.4	0.3 (1.0)	0.4 (1.0)
*C30 (7255/7173)	0.3	0.4 (1.0)	0.3 (1.0)

Jurkat HP21 cells were transfected with pGEM(cat) plasmid DNA containing the MMTV segments indicated. Cells were treated with 10 ng/ml PMA and 30 mg/ml Con A, without or with 100 ng/ml CsA for the final 12 hr of a 42 hr culture period. Each CAT assay contained 350 mg protein. pRSV(cat) was used as a constitutive, CsA-insensitive control. (*) indicates samples which were tested in a separate experiment using the same experimental conditions. Data are given as the percent of total substrate acetylated, with the numbers in parentheses indicating the activity relative to that obtained with untreated cells.

Table 4-2B. Activation of CAT transcription by segments of the MMTV envelope gene

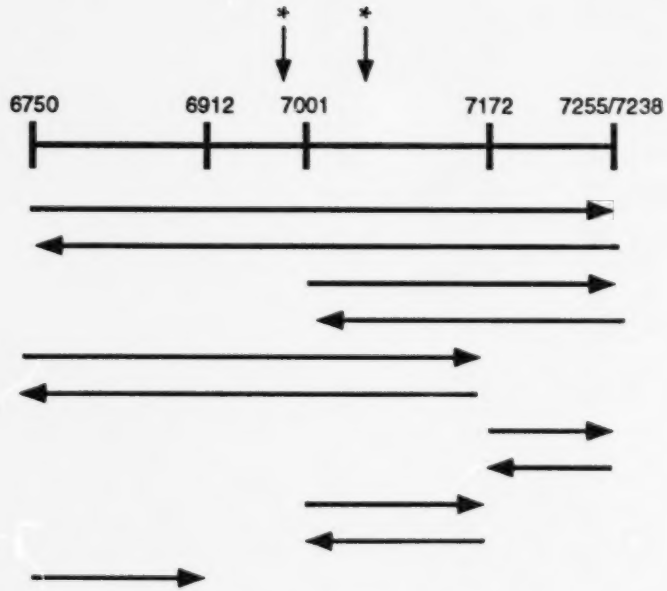
Promoter/enhancer	Untreated	Treated	Treated/CsA
pRSV	48	58 (1.2)	52 (1.1)
15-8 (6750/7328)	0.6	75 (136)	11.3 (20.5)
15-8 (7173/7328)	0.5	0.4 (1)	0.6 (1.3)
15-8 (7328/7173)	0.6	0.6 (1.0)	.96 (2)
15-8 (6750/7172)	0.3	33 (130)	7 (27)
C11 (6750/7255)	0.5	55 (109)	21 (42)
C11 (7255/6750)	0.3	40 (133)	5.3 (18)
C11 (7002/7255)	0.3	0.3 (1)	0.5 (2)
C11 (7255/7002)	0.4	5.8 (15)	0.5 (1.4)
C11 (6750/7172)	0.5	22.7 (49)	1.9 (4)
C11 (6750/6912)	0.5	0.4 (1)	0.3 (1)
C11 (7002/7172)	0.4	0.6 (1.5)	0.3 (1)
C11 (7172/7002)	0.4	0.3 (1)	0.4 (1.0)
C30 (6750/7255)	0.4	41 (102)	0.5 (1)
C30 (7002/7255)	0.5	10 (20)	0.8 (2)
C30 (7255/7002)	0.4	1.5 (4)	0.7 (2)
C30 (6750/7172)	0.4	17 (47)	0.4 (1.0)
C30 (7172/6750)	0.4	37 (80)	1.0 (2)

Jurkat HP 21 cells were transfected with pGEM(cat) plasmid DNA containing the MMTV segments indicated. Cells were treated with 15 ng/ml PMA plus 1.5 mM ionomycin without or with 100 ng/ml CsA for the final 12 hr. of a 42 hr. culture period. Each CAT assay was incubated for 3 hr. and contained 250 mg protein. pRSV(cat) was used as a constitutive, CsA-insensitive control. Data are given as the percent of total substrate acetylated, with the numbers in parentheses indicating the activity relative to that obtained with untreated cells.

Table 4-2C. Activation of CAT transcription by segments of the MMTV envelope gene, summary of results

The META fragment is shown and nucleotide positions corresponding to several restriction enzyme sites in the various META clones are indicated (6750-Sst I; 6912-Rsa I; 7001-Rsa I; 7172-Hinc II; 7255-Dpn I). The putative start site of META transcription occurs at position 7247. Several sequences were identified which may be important for regulation of META activity. These sites are listed in Table 4-4 and are positioned in the region indicated by (**). The arrows indicate the size, and orientation of the subfragments with respect to the CAT coding region. The table lists the various META clones and their source, and is a summary of experiments presented in Table 4-2A and 4-2B. CAT activity is graded +, ++, or +++ in increasing order of magnitude. Sensitivity of META activity to CsA suppression is rated as Y-yes; N-no; or P-partial. ND-not done.

Table 4-2C



EL4.E1- C30		12.1.19- C11		BALB/c- 15-8	
CAT activity	CsA sup.	CAT activity	CsA sup.	CAT activity	CsA sup.
+++	Y	+++	P	+++	P
-	-	++	P	ND	ND
+	Y	-	-	ND	ND
+/-	N	+/-	-	ND	ND
++	Y	++	P	++	P
+++	Y	ND	ND	ND	ND
-	-	-	-	-	-
-	-	-	-	-	-
ND	ND	-	-		
ND	ND	-	-		
ND	ND	-	-		

META element. RNase protection experiments shown in Figure 4-7 of pGEM2(cat)C30(6750/7255) indicated that transcripts may be initiating from within the vector at sequences just downstream of the META insert. Although conclusions cannot be drawn concerning the capacity of any of the META segments tested to function as start sites for transcription, what is evident is that the META element contains strong transcriptional activator sequences. Of the segments which were active, all required activation of the T-helper cell. Sequences lying between positions 6750 and 7172 resulted in strong inducible expression of CAT activity. This segment likely contains multiple regulatory elements, as has been shown for other conditional promoters like the IL2 upstream region and the MMTV 5' LTR. Regulation of META is likely complex. Linkage of some of these fragments to a heterologous promoter would contribute to localization of transcriptional activator elements.

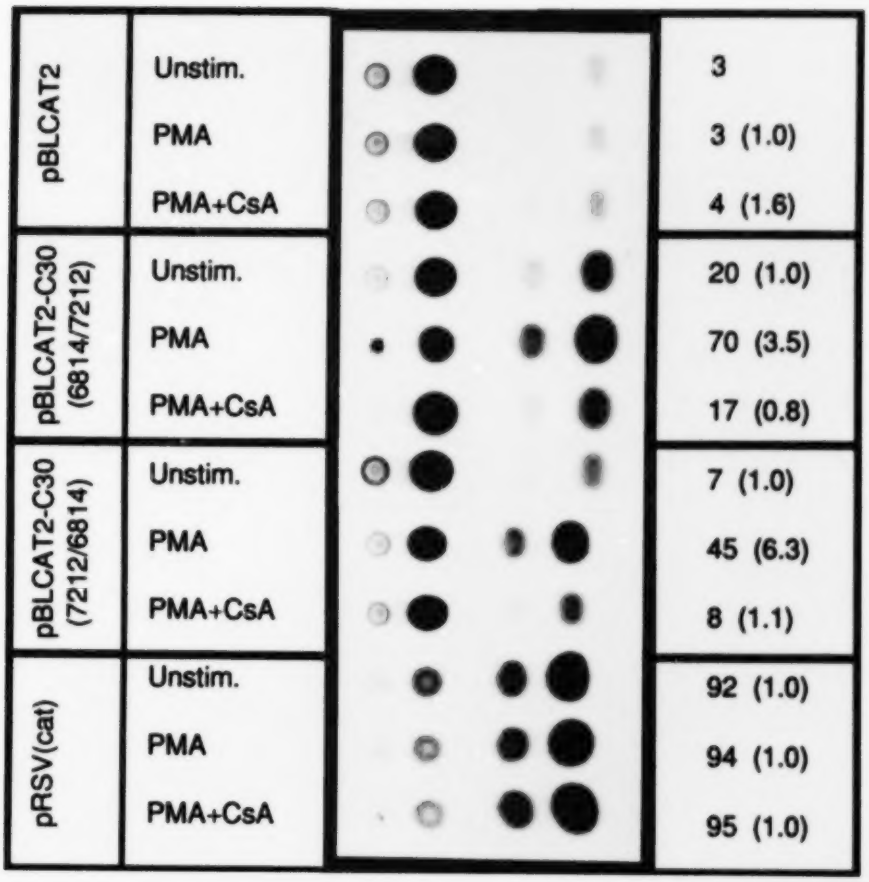
Activation of a heterologous promoter

Experiments were carried out to determine if the cloned META sequences contain elements which can control the activity of a heterologous promoter. We used the vector pBLCAT2, which contains the Herpes Simplex Virus thymidine kinase (tk) promoter, upstream of the CAT coding sequence. To remove any contribution or interference by the nominal start site within the original META fragment, the C30 fragment was copied with PCR primers Mtv.A11 and Mtv.S11 (Table 2-1). The antisense primer replaced the TATA sequence and copied only the sequence to the left (upstream) of it, thus removing the transcriptional start site at 7247. The copied fragment, containing MMTV sequences from 6814 to 7212, was shorter by 64 bp at the 5' end and 43 bp at the 3' end than C30(6750/7255).

Recombinant plasmids were transfected into EL4.E1 cells, which were treated with PMA, or with PMA and CsA, for the final 12 hr. of culture. Results are shown in Figure 4-8. The tk promoter alone (pBLCAT2 vector) induced a low, but detectable level of CAT expression, but this activity was insensitive to activation by PMA or suppression by CsA. Insertion of the META-derived fragment C30(6814/7212) upstream of the tk promoter increased the level of CAT expression by about seven fold even in unstimulated cells, and was further increased 3.5-fold upon activation with PMA.

Figure 4-8 The MMTV *env* gene fragment between positions 6801 and 7212 is an orientation-independent, CsA-sensitive transcriptional activator

C30(6801/7212) (forward orientation) and C30(7212/6801) (reverse orientation) were inserted into pBLCAT2 and used to transfect EL4.E1 cells. Cells were either left untreated, or were treated with 15 ng/mL PMA or with PMA and 100 ng/mL CsA during the final 15 hr of a 42 hr incubation period. The percent of acetylated product is shown in the right column, with numbers in parentheses indicating the level of activity relative to that obtained with untreated cells.


pBLCAT2
Unstim.
PMA
PMA+CsA
3
3 (1.0)
4 (1.6)
pBLCAT2-C30 (6814/7212)
Unstim.
PMA
PMA+CsA
20 (1.0)
70 (3.5)
17 (0.8)
pBLCAT2-C30 (7212/6814)
Unstim.
PMA
PMA+CsA
7 (1.0)
45 (6.3)
8 (1.1)
pRSV(cat)
Unstim.
PMA
PMA+CsA
92 (1.0)
94 (1.0)
95 (1.0)

The inducible increase, although not the constitutive level, was completely suppressed by CsA.

When placed in the reverse orientation with respect to the tk promoter, the same fragment also enhanced constitutive expression, although only 2.3-fold compared to pBLCAT2 lacking an MMTV DNA insert. Upon activation, however, CAT activity was increased by about seven fold, and this increase was also completely blocked by CsA. Therefore C30(6814/7212) MMTV DNA acts as an inducible, orientation-independent transcriptional activating sequence when linked to the heterologous tk promoter.

Further experiments with homologous segments of clones C11 and 15-8 yielded a similar pattern of results, that is, orientation-independent transcriptional activation of the tk promoter (Table 4-3). The segment (6801/7217), derived from C11 or 15-8, when placed in either orientation with respect to the CAT coding sequence in pBLCAT3 which lacks the tk promoter, did not result in significant increase in activity in PMA-treated EL4.E1 cells, indicating that this segment does not function efficiently as a promoter. When placed in the reverse orientation in pBLCAT3 both clonal isolates enhanced constitutive expression from the tk promoter by approximately 2.5 fold. Stimulation of transfected cells with PMA resulted in an increase of 6.5 and 11.6 fold with 15-8 and C11 respectively. Most of the PMA induced increase was suppressed by CsA (75 and 91% for the 15-8 and Cll, respectively). Lack of complete suppression by CsA was not unexpected, in that activity of the META elements C11(6750/7255) and 15-8(6750/7328) in the pGEM(cat) plasmids were not completely suppressed by CsA either.

Identification of start site of CAT in META linked to a heterologous promoter.

The origin of the CAT mRNA generated from the heterologous *tk* promoter was determined by RNase protection experiments using the pBLCAT2-C30(7212/6814) plasmid as the source of the induced mRNA. This construct contains the META fragment in the reverse orientation, making it possible to examine expression in EL4 cells as well as Jurkat. The radioactive probe RNA was synthesized from the vector pCM1 (see Figure 2-3). It was hybridized to mRNA from either EL4.E1 or Jurkat cells which had been transfected with pBLCAT2-C30(7212/6814), and stimulated with ionomycin plus PMA. This combination was found to induce expression of the META

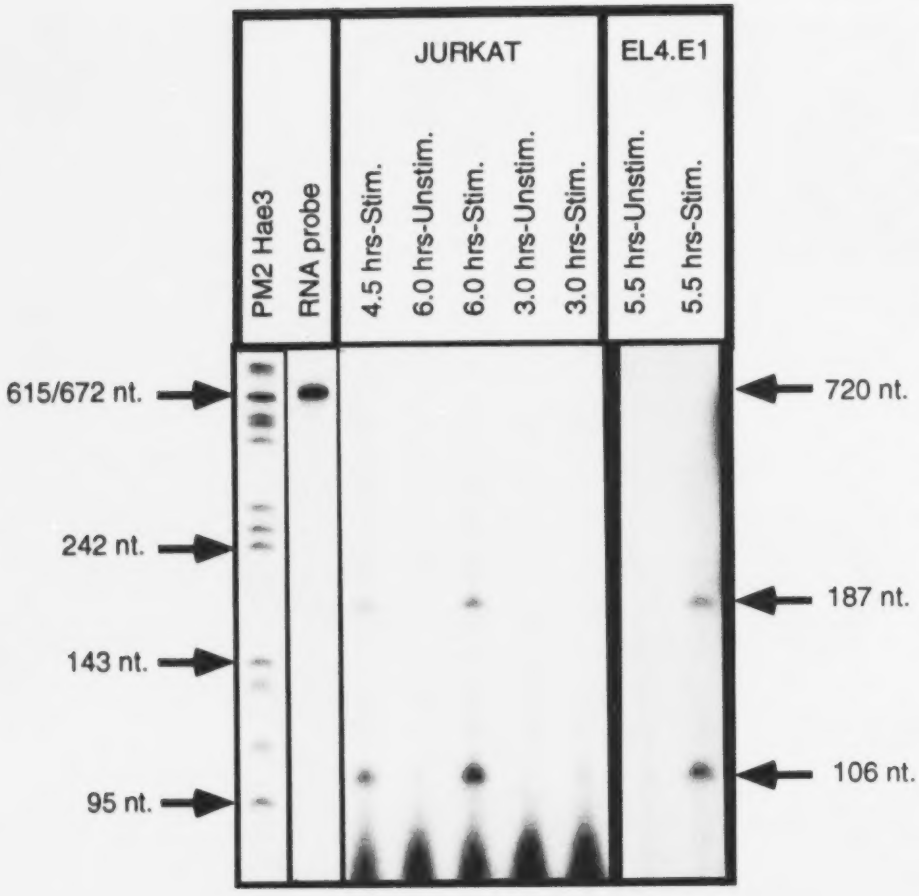
Table 4-3. Activation of the heterologous tk promoter by segments of the MMTV envelope gene

Plasmid	Promoter*MMTV segment	Untreated	Treated	Treated/CsA
pRSV(cat)	pRSV	80	77 (.96)	81.7 (1.0)
pBLCAT2	ptk*none	1.9	2.7 (1.4)	3.1 (1.6)
pBLCAT3	none*none	.37	.49 (1.3)	.48 (1.3)
pBLCAT2	ptk*15-8(7212/6801)	5.1	32.6 (6.4)11.7	
(2.3)				
pBLCAT3	none*15-8(6801/7212)	.33	0.8 (2.4)	.47 (1.4)
pBLCAT3	none*15-8(7212/6801)	0.8	4.4 (5.4)	2.5 (3.1)
pBLCAT2	ptk*C11(6801/7212)	2.2	18.9 (8.6)	ND.
pBLCAT2	ptk*C11(7212/6801)	5.9	68.4 (11.6)12.1	
(2.0)				
pBLCAT3	none*C11(6801/7212)	.31	.48 (1.6)	.34 (1.1)
pBLCAT3	none*C11(7212/6801)	.38	1.0 (2.6)	.38 (1.0)

EL4.E1 HP8 cells were transfected with plasmid DNA using the DEAE-dextran procedure. pBLCAT3 does not contain a defined promoter whereas pBLCAT2 contains the *tk* promoter 5' to the cat coding sequence. The MMTV env gene segment used is indicated. Cells were treated with 15 ng/ml PMA, without or with 100 ng/ml CsA for the final 12 hr of a 45 hr culture period. Each CAT assay contained 87.5 mg protein and the assay was incubated for 3 hr instead of the standard 4 hr Data are given as the percent of total substrate acetylated, with the numbers in parentheses indicating the activity relative to that obtained with untreated cells.

Figure 4-9 Start site of CAT mRNA generated from a pBLCAT2-META construct

EL4.E1 HP2 cells and Jurkat cells were transfected with pBLCAT2-C30(7212/6814). Following incubation for 20 hr, cells were treated with 15 ng/mL PMA and 1.5 μM ionomycin for the times indicated. Total RNA was isolated and used for the RNase protection assay. The RNA probe was produced from plasmid pCM1 (Figure 1-3), and was of length approximately 720 nucleotides (right hand lane). Transcripts initiating from the tk promoter should be 196 nucleotides long, essentially the same as the longer of the two protected fragments shown in the figure. The shorter protected fragment, of length 103-106 nucleotides, likely corresponds to probe-RNA hybrids which were cleaved within an A-rich region present between position 7 and 13 with respect to the translational start site of the CAT coding region. This artifactual cleavage has been described elsewhere (72). The markers were derived by labeling PM2 DNA (^{32}P) which had been cut with Hae III.


JURKAT
EL4.E1
PM2 Hae3
RNA probe
4.5 hrs-Stim.
6.0 hrs-Unstim.
6.0 hrs-Stim.
3.0 hrs-Unstim.
3.0 hrs-Stim.
5.5 hrs-Unstim.
5.5 hrs-Stim.
615/672 nt.
242 nt.
143 nt.
95 nt.
720 nt.
187 nt.
106 nt.

constructs even more strongly than did Con A plus PMA, as has also been found for lymphokine gene transcription.

The results of the RNase protection experiments are in Figure 4-9. Transcripts initiating from the tk promoter should be 196 nucleotides long, essentially the same as the longer of the two protected fragments shown. The shorter protected fragment, of length 103-106 nucleotides, likely corresponds to probe-RNA hybrids which were cleaved within an A-rich region present between position 7 and 13 with respect to the translational start site of the CAT coding region. This artifactual cleavage has been described previously (72). Both fragments were increased in Jurkat and EL4.E1 cells following stimulation. We did not detect tk-CAT mRNA in unstimulated cells, because the sensitivity of the method was too low to detect the resulting mRNA.

These results show that the origin of CAT mRNA is at, or close to, the transcriptional start site in the tk promoter. Induction for various times led to an increase of RNA which initiated at this site, in keeping with the results of the CAT assay itself. We conclude that a 400 bp portion of the META element, derived from three different cellular sources, can act as an inducible, orientation-independent and CsA-suppressible enhancer-like element in T-helper cells.

Comparison of META sequences

Nucleotide sequences for clones C30(6750/7255), C32(6750/7255), C23(6750/7255), C11(6750/7255), and 15-8(6750/7328) are compared to the proviral form of the milk-borne virus (138) in Figure 4-2. Clones C30, C23 and C32 are derived from EL4.E1 cells, C11 from a T cell hybridoma and 15-8 from BALB/c spleen cells. All clones with the exception of C23 are functional transcriptional activator elements in stimulated T helper cell lines. There are 15 point differences between C30 and C23, some of which presumably account for differences in their respective behavior. Clone 15-8, is the most similar to the MMTV milk-borne sequence, differing at 13 of 505 bases (i.e., less than 3% of the total META element). The META clones contain binding motifs which are present in most eukaryotic promoters. There is a TATA box located some 30 bases upstream of the start site of the META-regulated transcript at position 7215 and a CCAAT sequence at 7196.

TABLE 4-4 Comparison of META sequence with known binding motifs

Consensus Sequence	Binding protein/ Site	Location	MMTV position	Ref.
GGGGTTTAAAGA GGGGTTTAAAGA GGGTTTAAAaA	AP-3/TCEp	mIL2, -173 to -162 hIL2, -163 to -152	6976-6986 (C30)	174
CTGTGGTAA gTcTGGTAA	CBF/MoLV core	MoLV enhancer	6990-6998	182
ATGTAAAA ATGTAAAA	Oct-1/ Oct-1 motif	NFIL2A hIL2, -66 to -90	7001-7008	47
TGTGG AAAGTC TGaGGggAAGTC	AP-3/SV40		7008-7019	129
CGCCATTTT CcCCATTTg	UCRBP/UCR	MoLV LTR	7029-7036	182
TTTTCCTCTT cTTTCCTtcT	Purine Box (Pu box)	mIL2, -120 to -145 mIL2, -264 to -292	7039-7048	174
TTCCT TTCCT cTCCT TTtCT	Ets-1 site	mIL2, -120 to -145 mIL2, -264 to -292 hIL2, -145 to -158 hIL2, -264 to -284	7041-7045 7047-7051 7052-7056	190
AAAGAAATTCC AAAGAAgTTgC	CD28 complex/ CD28RO	hIL2 -164 to -154	7221-7231	62

There are several elements which are similar to cis-acting regulatory elements localized within a region of 300 bp immediately upstream of the IL2 gene, and which may account in part, for the induction of the META transcript. The positions of putative binding sites are shown in Figure 4-2 and are listed in Table 4-4. A TCEp-like segment (174) is centered at 6980 (-266 relative to the start of transcription). This binding motif in the IL2 gene (at -172 in the mouse gene) shares binding with AP-3 elements, but upon induction of EL4 cells with PMA, a separate factor (other than AP-3) binds to it (174). There is also an AP-3-like motif centered at position 7013. The AP-3 core sequence from the SV40 enhancer was used to purify AP-3 (129) and the corresponding oligonucleotide has been used in mobility shift assays to compete for protein binding to the NF-κB site in the IL2 enhancer (78). CsA and FK506 treatment of stimulated T-cells causes a moderate decrease in AP-3 binding to the SV40 AP-3 binding site (52, 78).

The IL2 gene contains two octamer binding motifs within the NF-IL2A and NF-IL2D response elements (47). Oct-1 and associated proteins have been shown to bind to the NF-IL2A binding site (also called antigen-receptor response element or ARRE-1)(199). Mutation of the octamer motif within this site significantly reduces the activity of the IL2 promoter in Jurkat cells treated with PMA and calcium ionophore (47, 199). Induced transcription driven by NFAT-1 and NF-IL2A is blocked by FK506 or CsA (10, 52).

The block of purines at META (7039 to 7048) is similar to the conserved purine block found in the mouse (174) and human IL2 genes, and in a number of other cytokines (66). In IL2, the purine block is within a larger segment (not present in MMTV) defining the critical NFAT-1 site, which is of primary importance in regulating IL2 transcription (178). The NFAT-1 and the NFIL2-B sites within the mouse and human IL2 promoter also contain purine-rich motifs that correspond to the consensus sequence for members of the *ets* proto-oncogene family (190). Ets-1 specifically interacts with regulatory sequences of several genes including the T-cell receptor α gene enhancer (90, 190) and the LTR of Moloney murine sarcoma virus (81). There are three segments which are similar to the Ets-1 recognition sequence within the MMTV *env* gene in the region we have defined as META. One of the three copies, situated between 7041 and 7056 has identity with the Ets-1 consensus sequence. Stimulation of human T-cells through

the CD28 surface antigen induces binding of a protein complex to site called the CD28-responsive complex (CD28RC) situated between -154 and -164 of the human IL2 enhancer (62). A similar sequence is located in the META element between positions 7221 and 7231, which lies downstream from TATA and is not present in the tk constructs.

There are sequences within the MMTV *env* gene which have some homology to regulatory elements present in the LTR of Moloney leukemia virus (MoLV). This virus causes T-cell lymphomas and leukemias in rats and mice. A sequence related to the MoLV core sequence is positioned between 6990 and 6998 and an Upstream Conserved Region (UCR)-like motif starts at 7029. Elements within the MoLV LTR enhancer contribute to induction of oncogenesis and disease specificity (182, 183). Mutations within the MoLV core and adjacent elements reduced the incidence of thymic leukemias and resulted in an corresponding increase in erythroleukemias (183). The UCR core sequence binds ubiquitous nuclear factors and contributes to the negative regulation of the MoLV promoter (206). There is no sequence variation between the META clones within the UCR-like region.

CHAPTER 5. SUMMARY DISCUSSION

Summary of results

It was previously shown by this laboratory, and has been confirmed in this study, that there is a functional promoter within the MMTV proviral *env* gene in EL4.E1 cells. Transcription from this promoter initiates at or near position 7247 (51, Figure 2-3). Based on Northern blot analysis (Figure 3-2), it was determined that META transcripts were detectable within two hours of EL4.E1 activation by PMA and that accumulation peaked at about nine hours. The temporal fluctuations in META transcription paralleled that of IL2 gene expression in these same cells and expression of both mRNAs was blocked by CsA.

To follow-up these preliminary studies, a representative sampling of the right hand end of the MMTV provirus was cloned using the PCR technique. Segments corresponding to 6750/9901, isolated from genomic DNA from EL4.E1 cells and from a T cell hybridoma, 12.1.19, were cloned into the plasmid pGEM3Z. A 505 bp fragment (6750/7255) isolated from a number of these clones was inserted into an expression plasmid, pGEM2(cat) upstream of the coding region for CAT. The clones C30, C23, and C32 were derived from EL4.E1 cells and clone C11 from 12.1.19 cells. A segment, clone 15-8(6750/7328) from BALB/c splenic DNA, was also inserted into pGEM2(CAT). These MMTV recombinant plasmids were introduced by the DEAE-dextran transfection protocol into a variety of cell types. These transient-expression studies yielded a number of intriguing results.

With the exception of C23(6750/7255), all clones yielded strongly inducible CAT activity in activated T helper cell lines. The ability of CsA to suppress this activity was variable. For example, the activity of C30(6750/7255) was completely blocked at the same CsA concentration which reduced the activity of C11(6750/7255) by 60-70%. The basis for this variability presumably resides in sequence differences between various clones. C11(6750/7255) and C30(6750/7255) were tested in a variety of cell types including T helper cells lines which require differing stimuli to induce cytokine production. C30(6750/7255) or C11(6750/7255) were active in EL4.E1 cells treated with PMA, and in Jurkat cells treated with either Con A and PMA, or PMA and ionomycin. They were also active in 12.1.19 cells treated

with either Con A or anti-CD3ε. PMA alone was not sufficient to induce CAT activity in either Jurkat or 12.1.19 cells. Both META elements were inactive in HeLa cells, in two B cell lines, in the mastocytoma P815, and in a CTL cell line. Therefore META appears to be active only in T helper cells which have been treated with the agents which induce cytokine production. As a corollary statement, induction of META activity appears to be specific to T helper cells and to their state of activation as opposed to the inducing agent used. That is, various agents stimulate its activation in T helper cell lines, but agents which worked in these cells were without effect in other types of cells. These data also imply that the conditional expression from META in EL4.E1 cells is responsive to a signaling pathway similar or identical to that of the IL2 gene.

In addition, the fragment (6814/7212) was excised from clones C30, C11, and 15-8 and inserted into a CAT expression plasmid containing the *tk* promoter. This 400 bp portion of META behaved as an inducible, CsA-sensitive, orientation-independent enhancer in activated EL4.E1 cells.

Correlations between IL2 and META gene expression

We have suggested that the expression of META activity mimics the transcriptional regulation of the IL2 gene. There are several mechanisms involved in controlling the levels of IL2 gene expression including changes in transcriptional rate, termination of transcription and mRNA stability. The importance of one or more of these mechanisms involved in regulation of META transcript requires further investigation.

Data from deletion analysis of the META segment (presented in Chapter 4) did not allow us to pinpoint regions which contain elements required for the restricted expression of META. Equivalent levels of CAT expression were not obtained with the various subfragments of META derived from the different clones. Linking of the subfragments to a heterologous promoter and testing for their enhancer activity in stimulated T helper cell lines may yield additional information.

Sequence analysis of the META clones did reveal the presence of sites which have homology to regulatory elements within the IL2 gene. These include a purine-rich block at 7039/7048 which is similar to a portion of the binding site for the factor NF-AT, and an Oct-1 site at 7001/7008. The ability of NFAT and NFIL2-A (which contains an Oct-1 site) to direct transcription

is strictly dependent on signals generated through engagement of the TcR. In addition, in Jurkat cells, this signal can be mimicked by agents which activate PKC (PMA) and modulate Ca^{++} levels (calcium ionophores) (195). CsA inhibits transcription activated by multimers of the NFAT and NFIL2-A sites (10, 52, 78). We have shown that META activity can only be fully induced in Jurkat cells using a combination of PMA and either ionomycin or Con A, and all clones were at least partially sensitive to CsA. I conclude that activation of META is in part due to the binding of regulatory proteins present as a consequence of T cell activation.

The META fragment also contains two AP-3-like motifs at 6976/6986 and 7008/7019. As is the case for the IL2 gene (87), regulation of META probably depends on both induced regulatory proteins and constitutively expressed ones.

There is a sequence similar to the CD28RC binding site at position 7221 to 7231. Binding of a CD28 responsive complex may not be involved in META regulation as META segments (6750/7172) which do not contain this region were fully inducible in activated Jurkat cells and were CsA-sensitive. We have not tested induction of META activity in T cells stimulated through the CD28 pathway.

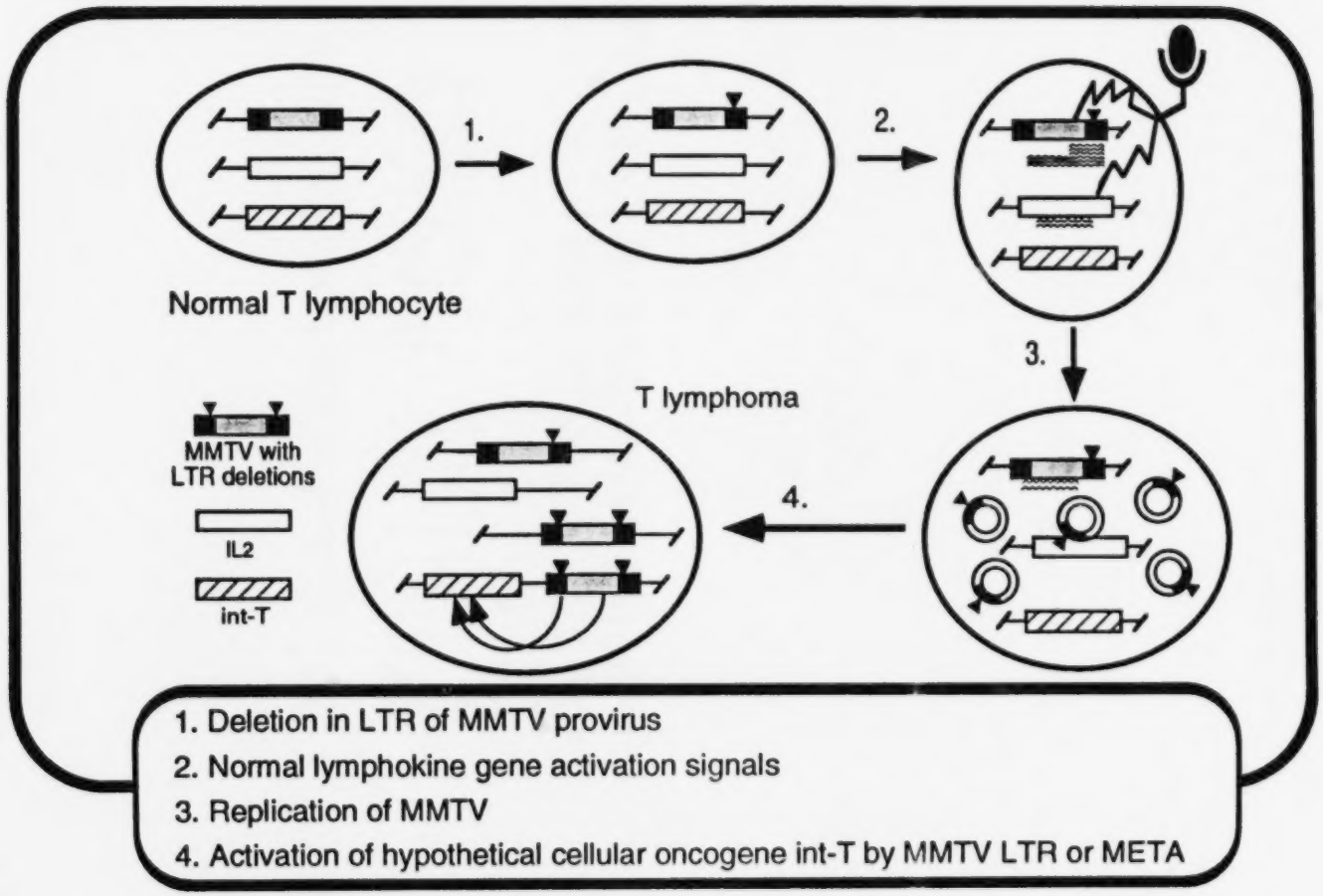
META also contains regions which resemble portions of the MoLV enhancer in the segment (6990/7029) The 72 bp enhancer within the MoLV LTR contributes to induction of oncogenesis and determination of disease specificity (83, 182, 183). Further analysis is required to determine if similar sequences in META contribute to expression in T cells.

Regulation of transcription by META will likely prove to be complex and may involve both known and novel regulatory proteins. Studies are currently underway in this laboratory to determine which sites and cognate binding proteins are important for the inducible, CsA-sensitive expression of META in T cells.

A model of MMTV-induced lymphomagenesis

Although there is no direct proof that MMTV is a causative agent of T lymphoma, there is a large body of correlative data, as outlined in Chapter 1. I will propose a model for MMTV involvement which begins with the fact that the virus does not encode an known oncogene. The main points of this model are as follows (see Figure 5-1).

Figure 5-1 Model of MMTV-induced lymphomagenesis



1. A deletion occurs in the U3 region of MMTV provirus in a T cell. The mechanism for this is unknown. This deletion may occur in the 3' LTR in an integrated copy of MMTV, or may originate as a result of a mutational event occurring during reverse transcription of the MMTV RNA genome into a DNA replicative intermediate.
2. The deletion-containing MMTV provirus is transcribed into genomic RNA in an activated T cell and additional proviral copies are produced. The deletion in the LTR and the META element contribute to T tropic expression of the MMTV provirus. The activated state of the T cell facilitates the reintegration and amplification of MMTV provirus into the chromosome of the preleukemic cell.
3. One or more of the newly-synthesized provirus integrates in the vicinity of a cellular proto-oncogene, analogous to the mechanism of MMTV-induced mammary adenocarcinoma generation.
4. When the T cell is activated, enhancer elements within the altered LTR and/or in META induce the expression of a cellular proto-oncogene, thereby contributing to T lymphomagenesis.

The role of exogenous virus in T lymphoma

It is not possible to establish with certainty the original source of the amplified MMTV provirus in T lymphoma cells, but infection with exogenous virus may increase the incidence of T lymphoma development. In GR mice, T lymphomas develop in male mice at an incidence of 20% when newborns are nursed on viremic mothers (134). The frequency decreases to 8% when offspring receive virus-free milk from foster mothers. The *Mtv-2* loci of GR mice is responsible for virus production in this strain. Amplified copies of the Mtv-2 loci, are present in primary tumors and were found to be genetically stable during passage in vivo and in vitro (134). The MMTV-variant thymotropic virus DMBA-LV induces thymic lymphomas with a short latency period. By 17 days of infection, thymic cells contain proviral DNA and by 40 days all contain new proviral copies. Some of the tumors are oligoclonal suggesting that amplification occurred subsequent to the initial infection (8).

Racevskis concluded that the amplified MMTV provirus in EL4 cells might have originated from a horizontally transmitted exogenous virus (155). This conclusion was based on the observation that the majority of

amplified provirus in EL4 have a BamH I site within the *env* gene at position 7536 which is lacking in the three Mtv loci associated with the C57Bl/6 parental cell line. This restriction site is present in the milk-borne virus of C3H mice (138) and in the endogenous forms of *Mtv-1* and *Mtv-2*, both shown to be capable of producing infectious virus (137, 134). In this study, sequence analysis of the 2.7 kb fragments (6750/7255) from the C30, C32 and C23 clones, derived from EL4.E1 cells confirmed that all had the 494 bp deletion at the same location and with the same endpoint sequence (data not shown). C23 and C32 have a BamH I restriction site at 7536 which was lacking in C30. It is likely that C30 reflects the sequence of a provirus which resulted from the same original amplification, although it lacks this restriction site due to further mutation, as has been documented in other tumors arising in C57Bl/6 mice (42).

There is a significant difference in the response of C3H and GR mice to infection with MMTV. Both develop mammary adenocarcinoma with a high frequency, but T lymphomas with amplified, altered MMTV have been reported only for the GR strain. The basis for this difference is not known, but may result from differences in response of Vβ14$^+$ T cells which recognize the relevant MMTV-induced Mls antigen. As evidence that such a difference exists, it is noteworthy that when the two strains were exposed to the Mls antigens by transgenic introduction, C3H Vβ14$^+$ T cells were efficiently deleted (74), but the homologous GR T cells were deleted only incompletely, over a period of 4-5 months (2). Whether this is related to the difference in T lymphoma incidence, or whether other mechanisms are responsible, is not known. One possible explanation is that fewer T cells in the C3H strain are potential candidates for transformation due to: 1. efficient deletion in the thymus early in T cell ontogeny in response to exposure to the Mls antigen 2. establishment of an anergic state to the Mls antigen of peripheral T cells. Therefore activated T cells capable of becoming infected with MMTV would be present at a lower frequency in the C3H mouse compared to the GR mouse. Do T lymphomas develop because the infected T cells have TcR reactivity for the Mls antigen presented by infected APC? This question can only be answered by analysis of the Vβ genes utilized by the tumor cells.

Amplification of MMTV in T cells

It is likely that the deletion within the U3 region of the LTR occurs prior to amplification of the MMTV provirus. As discussed in Chapter 1, T lymphomas expressing MMTV share several common features including amplification of MMTV provirus and deletions within the LTRs. These deletions, unique to each tumor are of a similar size and location (133, 51, 120, 9, 93). Because the deletion is present in both proviral LTRs, it is likely that the mutation occurred first in the U3 region of 3' LTR and was duplicated in the 5' LTR during reverse transcriptase synthesis of the positive DNA strand (Figure 1-2). It can also be argued that expression and amplification of a deletion-containing MMTV provirus likely occurs within the same preleukemic cell, considering the clonal nature of the deletion within the LTR. By this mechanism an integrated MMTV provirus containing altered LTRs would be expressed, and the genomic length RNA would then be reverse-transcribed into a DNA proviral form prior to integration at different chromosomal locations.

An analogous mechanism may occur during the development of AIDS. The human immunodeficiency virus type 1 (HIV-1) can be found as extrachromosomal DNA in peripheral lymphocytes of asymptomatic patients. These latent forms retain the ability to integrate upon T cell activation in vitro (23). T lymphoma cells expressing MMTV contain precursor proteins but have not been shown to produce infectious virus (131). Ringwold and coworkers have detected non-integrated MMTV DNA molecules in rat hepatoma cells infected with MMTV(163a). It has also been shown that in a C57Bl/6 lymphoma, several of the additional MMTV provirus were flanked by the long interspersed retrotransposon L1Md at a frequency higher than would be predicted for random integration (42). The author suggested that both MMTV and L1Md integrated as retrotransposon-like elements into transcriptionally active regions of the host chromosome.

The deletions within the U3 region of the LTR may facilitate the increased expression of the MMTV proviral DNA in the preleukemic T cell prior to and following amplification of unintegrated DNA intermediates. Evidence detailed in Chapter 1 indicates that deletions occurring in the U3 region may remove NREs and/or create novel enhancer elements and contribute to T cell tropic expression (120, 133, 135, 188, 214). It should also be

emphasized that the thymotropic MMTV-related virus, DMBA-LV also has a altered LTR compared with wild-type MMTV (9).

In the present model, the META sequence contributes to the initiation of MMTV proviral synthesis. As demonstrated, activation of the T cell by signals delivered through the TcR activates the META element. This may promote opening of chromatin structure in the vicinity of META thereby increasing accessibility of transcription factors to the 5' LTR promoter. In other words, it is the enhancer activity of META which is critical. Transcription from the *env* gene start site may or may not need to occur in such a mechanism.

There is no evidence indicating that the META sequence functions as a biological promoter in cells other than PMA-treated EL4 cells, although the possibility should not be excluded. The META transcript includes the coding region for the Mls determinant (ORF protein) in the 3' LTR. The ORF protein has never been detected in primary tissue cells implying that levels of mRNA may be low, or expressed infrequently. It would be of great interest to determine the start site of mRNA containing the *orf* gene in immune cells presenting the Mls determinants to ascertain whether META is functioning in these instances as the predominant promoter.

In this study it was demonstrated that although clone C11(META), derived from 12.1.19 cells was a strong transcriptional regulatory element when tested in isolation, there was no evidence to indicate that META was endogenously active in these cells. Lack of detectable activity could be due to the insensitivity of detection methods or could reflect transcriptional silence due to chromosomal positioning or hypermethylation of the loci. This lack of endogenous META activity could also be due to down-regulation by elements contained in the LTR or elsewhere. As discussed in the introduction, the LTR does in fact appear to contain NRE (121, 136, 166).

The model predicts that newly-acquired copies of MMTV are inserted in the vicinity of cellular proto-oncogenes in a T cell. Activation of the T cell then activates META and/or enhancer elements in the deletion-containing LTR, and the increased expression of the proto-oncogene contributes to neoplasia. We have demonstrated that META is a strong, inducible, orientation-independent enhancer when linked to an heterologous promoter. It has not yet been determined which, or indeed whether cellular proto-oncogenes are deregulated as a consequence of MMTV insertion. It is plausible that ampli-

fication may disrupt more than one proto-oncogene. Coexpression of such genes and/or lack of expression of others may synergize to increase the likelihood of oncogenesis. For example, studies of slow transforming virus often implicate more than one proto-oncogene in tumor development (205a). Infection by MoLV of transgenic animals expressing c-myc linked to the immunoglobulin heavy chain enhancer results in a dramatic acceleration of lymphomagenesis. It was proposed that several identified genes cooperate in the observed oncogenesis (201).

In summary, I have isolated and characterized a powerful transcriptional activator from the *env* gene of MMTV. The results are consistent with the possibility that META could carry out one or more of the following activities: control of the transcription of Mls loci, enhancement of transcription from the 5' LTR in preleukemic T cells, and, following amplification of MMTV provirus, insertional mutagenic activation of cellular proto-oncogene(s). In addition to these as-yet unproven functions, the powerful, specific transcriptional activator in META has potential applications as a reagent for research, and in the development of novel therapeutic approaches. For example, META could be used to control the expression of an antisense transcript or a transcript encoding a deficient protein in T cells. The META element could potentially be used in vivo to limit the transcription of HIV-1 by activation-dependent synthesis of an appropriate antisense transcript in infected T cells.

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NOTE TO APPENDICES

Early in my thesis work, I was studying some of the phenotypic characteristics of murine IL2-dependent antigen-specific CTL cell lines (Type 1 CTL). It was previously shown by this laboratory that in the presence of antigen and high levels of IL2, these cells could be converted to adherent non-specific CTL (Type II CTL)(85a). Conversion could also be achieved by incubation with antigen and supernatants from PMA-stimulated EL4.E1 cells. Levels of IL2 in the supernatant were significantly lower than the levels of recombinant IL2 required to achieve conversion, suggesting that the PMA-treated EL4.E1 supernatant contained a factor involved in the conversion of a Type 1 CTL to a Type II CTL.

We obtained recombinant mouse IL4 from Dr. S. Gillis of Immunex Corporation. Although we did not identify a "conversion factor" several interesting results were obtained using the rmIL4 provided. These observations were reported in a manuscript titled "IL-4 potentiates the IL-2-dependent proliferation of mouse cytotoxic T cells".

We demonstrated that recombinant mouse IL4 synergizes with low levels of IL2 to increase the yield of cytotoxic activity in a primary MLR, and the proliferation of both cloned IL2 - dependent CTL lines and cells obtained from a primary MLR. IL4 did not induce the proliferation of any of several cloned CTL cell lines on its own. It also did not replace IL2 in stimulating the growth or reactivation of quiescent, antigen-dependent CTL clones. However, IL4 was synergistic with IL2 following reactivation of the quiescent cells with antigen plus IL2. Enhancement by IL4 of the IL2 - driven proliferation of an antigen - independent CTL line was blocked by the addition of anti - IL4 monoclonal antibody. Although incubation of the CTL clones with IL4 or with IL2 plus IL4 induced a transient increase in the expression of the mRNA encoding the 55kD IL2 receptor, no change in the number or affinity of IL2 receptors due to IL4 was detected (binding studies done by Dr. J. Hooton). This suggests that IL4 does not potentiate the IL2 response by altering IL2 receptor levels.

My involvement with the second manuscript, 'Development of pre-cytotoxic T cells in cyclosporine-suppressed mixed lymphocyte reactions' resulted from experiments in which I generated a spleen cell MLR in the presence of CsA. After 5 days the cells were harvested, washed and recul-

tured in rhIL2 for a further 48 hr. Surprisingly, although only low levels of lytic activity were detectable on day 5, after reculture there was significant lytic activity indicating that although CsA blocked the generation of cytolytic MLR cells, it did not inhibit the development of CTL precursers. In collaboration with Dr. J. Hooton and Cheryl Helgason, this in vitro system was further studied.

CsA blocked the generation of cytolytic activity in a primary MLR of mouse spleen cells. As expected from the known mechanism of action of this drug, it also blocked the accumulation of IL2 during the MLR. Addition of recombinant human IL2 did not overcome the inhibition of CTL generation, even when it was added daily to keep its level similar to that produced in a normal MLR. Daily addition was necessary, because the CsA-inhibited MLR consumed IL2 at a high rate. The outcome of a 5-day MLR in the presence of CsA (CsA-MLR) depended on whether or not IL2 was continuously present. In the presence of IL2, there was no generation of CTL activity, probably because such cultures contained IL2-dependent suppressive elements. However, when day 5 CsA-MLR cells generated in the absence of IL2 were washed and recultured with rhIL2, there was a burst of CTL activity, with a greater than 50-fold increase in alloantigen-specific cytotoxicity within 24 to 48 hours. This increase is not explainable simply by the proliferation of existing effector CTL. The non-cytotoxic cells produced in an MLR in the presence of CsA, and which can be rapidly activated to cytotoxic effector cells by IL2, are termed "precursor-effector CTL" (peCTL). They could be detected by day 3 of a primary CsA-MLR culture. Their conversion to effector CTL by IL2 was not inhibited by CsA. Exposure of peCTL to IL4 also generated CTL activity, to a somewhat lesser degree than IL2, but the IL4-induced activation was inhibited by CsA, suggesting that it depended on the induction of another CsA-sensitive lymphokine. The intracellular levels of mRNAs encoding the CTL-specific serine esterases CCP2 (granzyme C) and CCP1 (granzyme B) increased rapidly during the IL2-driven conversion of peCTL to effector CTL. This study demonstrated that CsA can lead to the generation of precursors for CTL, the peCTL, which can be rapidly converted to cytotoxic effector cells by IL2.

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0022-1767/90/1444-1331$02.00/0
THE JOURNAL OF IMMUNOLOGY
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Vol. 144, 1331–1337, No. 4, February 15, 1990
Printed in U.S.A.

IL-4 POTENTIATES THE IL-2-DEPENDENT PROLIFERATION OF MOUSE CYTOTOXIC T CELLS[1]

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In addition to the regulation of B lymphocyte growth and differentiation, the cytokine IL-4 (BSF-1) exerts effects on T lymphocytes and other bone marrow-derived lineages. We show here that recombinant mouse IL-4 synergizes with low levels of IL-2 to increase the yield of cytotoxic activity in a primary MLR, and the proliferation of both cloned IL-2-dependent CTL lines and cells obtained from a primary MLR. IL-4 did not induce the proliferation of any of several cloned CTL cell lines on its own. It also did not replace IL-2 in stimulating the growth or reactivation of quiescent, antigen-dependent CTL clones. However, IL-4 was synergistic with IL-2 after reactivation of the quiescent cells with antigen plus IL-2. Enhancement by IL-4 of the IL-2-driven proliferation of an antigen-independent line was blocked by the addition of anti-IL-4 monoclonal antibody. Although incubation of the CTL clones with IL-4 or with IL-2 plus IL-4 induced a transient increase in the expression of the mRNA encoding the 55 kDa IL-2 receptor, no change in the number or affinity of IL-2 receptors because of IL-4 was detected. This suggests that IL-4 does not potentiate the IL-2 response by altering IL-2 receptor levels. Instead, we propose that the synergistic effect of IL-4 is mediated by a different signalling mechanism from that used by IL-2.

IL-4 (BSF-1) was originally identified as a B cell growth and differentiation factor. It is produced by some T lymphocytes and possibly by mast cells (1). Receptors for this cytokine have been detected on both hematopoietic (2, 3) and nonhematopoietic cells (2), although its biological function in the latter is not understood. It is evident that IL-4 is a multifunctional cytokine that modifies the growth and behavior of various lymphoid and hematopoietic cells. It exerts effects on mast cell lines (4) and modulates the factor-dependent growth of committed erythroid, myeloid, and macrophage progenitor cells (5).

IL-4 also has effects on T lymphocytes, supporting the growth of some mouse and human T cell lines (6, 7) and of T cells that have been activated by antigen or mitogen (8, 9). It is an autocrine growth factor for a particular antigen-activated helper T cell clone (10) and may regulate the differentiation and proliferation of cytotoxic T lymphocytes. For example, when IL-4 was added to mouse (11) or human (12, 13) primary MLR cultures, it increased the level of cytolytic activity generated. In T cell ontogeny, Lyt 2$^-$/L3T4$^-$ thymocytes proliferate in response to IL-4 and phorbol esters without apparent differentiation (14). Carding and Bottomly (15) demonstrated that the proliferation of thymocytes incubated with IL-2 and submitogenic concentrations of PHA was enhanced by IL-4 and that the effect was restricted to Lyt 2$^+$/L3T4$^-$ cells. IL-4 in combination with lectin (16) or phorbol esters (17) induces CTL activity in a population of Lyt 2$^+$ murine splenocytes.

We have studied the effects of IL-4 on primary cells undergoing an MLR and on two phenotypically distinct kinds of cloned CTL lines. One of these is a type we have previously designated "type I" CTL (18). Type I cells are dependent on both IL-2 and antigen for long-term proliferation and for cytotoxic activity. They become quiescent in the presence of IL-2 but without antigen, and require both IL-2 and antigen for reactivation of growth and cytotoxicity from the quiescent state. Type I cells can be converted to antigen-independent growth, which we refer to as the type II phenotype, by incubation with high concentrations of IL-2 in the presence of either antigen or PMA as the second signal. They are also converted to type II by an activity that is present in a crude lymphokine preparation derived from stimulated EL4.E1 cells (18). Conversion to type II is irreversible. Here we present evidence that IL-4 enhances the IL-2-dependent proliferation of CTL generated in primary MLR cultures and of type I or type II cloned CTL, suggesting an accessory role for IL-4 in the expansion of activated CTL. Several lines of evidence will be presented that strongly suggest that IL-2 and IL-4 provide different signals to the CTL.

MATERIALS AND METHODS

Cytokines and antibodies. The source of all of the IL-2 used in this paper was recombinant human IL-2 secreted from yeast cells transformed with an expression vector containing a synthetic human IL-2 cDNA. The expression of the gene is from a yeast-mating type locus (19). Activity is expressed relative to a reference standard from Biological Resources Branch, NCI-FCRF (Frederick, MD). Half-maximal stimulation of T cell lines used in this work occurred between 1–5 U/ml of IL-2.

Recombinant, purified mouse IL-4 was provided by the Immunex Corp. (Seattle, WA). As described earlier (20), ~0.125 ng/ml of this purified material stimulates half-maximal proliferation of the HT-2 cell line. Thus, it has a potency in this assay of ~8000 ED_{50} units/μg of protein, where 1 ED_{50}/ml is required for a 50% response. The rat monoclonal anti-IL-4 antibody 11B11 initially produced by Ohara

Received for publication March 27, 1989.
Accepted for publication November 8, 1989.
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[1] This work was supported by grants from the National Cancer Institute and Medical Research Council of Canada.
[2] Recipient of a graduate studentship from the Alberta Heritage Foundation for Medical Research.
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and Paul (21) was kindly provided by Dr. Kim Bottomly (Yale University).

Animals. CBA/J and Balb/c mice were bred and maintained at the University of Alberta animal facilities and were used at 3 to 5 mo of age.

Cell lines. Cell lines were maintained in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 20 mM Hepes buffer, 0.1 mM 2-mercaptoethanol, antibiotics, and 10% fetal bovine serum (RHFM). The CTL cell lines were isolated as previously described (18). Briefly, spleen cells from CBA/J mice ($H2^k$) were cultured at a density of 1×10^6 cells/ml with an equal number of irradiated spleen cells from Balb/c mice ($H2^d$). After 5 days of culture, cells were grown in IL-2 for 48 h and cloned on a feeder layer of antigenic spleen cells. Antigen-specific CTL (type I) cloned by this procedure are dependent on both antigen and IL-2 for growth. For reactivation of quiescent type I cells, they were incubated for 72 h with 20 U/ml of IL-2 and a tenfold excess of irradiated antigenic cells. Type I cells remained viable and proliferated in IL-2 for up to a month, albeit at a diminishing rate.

Type I CTL were converted to a type II, which display nonspecific cytotoxicity and are antigen-independent for growth either by growth in high levels of IL-2, or by stimulation with a crude lymphokine preparation partially purified from stimulated EL4.E1 cells, as described elsewhere (18). In this study, MTL21.9(1) and MTL2.8.2 cell lines were used as representative type I and type II cells, respectively.

Bioassays. For proliferation assays, triplicate cultures were set up with the specified concentrations of lymphokines and 1×10^4 cells/0.2 ml in 96-well microtiter trays (Costar, Cambridge, MA). Incubation was carried out for 24 or 48 h at 37°C in 5% CO_2. Tritiated thymidine (0.25 μCi/well, specific activity 2 Ci/mmol, New England Nuclear) was present for the final 6 h. Cultures were harvested using a Skatron harvester (Lier, Norway) and radioactivity incorporated was quantitated by scintillation counting. Proliferation assays were done using the MTT[4] assay (22).

For cytotoxicity assay, S194 and P815 tumor cells ($H2^d$) were labeled with ^{51}Cr for 1.5 h. Typically, 2×10^6 cells were incubated in a 200-μl volume with 200 μCi of ^{51}Cr. After thorough washing, 1×10^4 target cells were incubated with doubling dilutions of effector cells in a 0.2-ml volume for 4 h at 37°C. Each determination was done in triplicate and the specific ^{51}Cr release was calculated as: % specific lysis = (experimental cpm − spontaneous cpm)/(maximum cpm − spontaneous cpm) × 100, where maximum cpm was the radioactivity released by detergent. Some results are expressed in lytic units (LU), where 1 LU releases 30% of the ^{51}Cr from the target cells under the conditions of the assay.

RNA isolation and Northern analysis. Total cytoplasmic RNA was isolated from samples of 2–3 × 10^6 CTL cells. The cells were washed in PBS, lysed with 1% Nonidet P-40, and the nuclei pelleted by centrifugation. The supernatant was incubated in 0.5% sodium dodecyl sulfate-0.1 mg/ml of proteinase K for 30 min at 37°C and the RNA was precipitated with sodium acetate (0.3 M, pH 5.5) plus 2 volumes of ethanol. RNA was analyzed by electrophoresis in 0.8% agarose-formaldehyde gels and transfered to Hybond-N nylon membrane (Amersham). Hybridization with the cloned probe for mouse 55 kDa IL-2 receptor chain was performed essentially as described earlier (23), with the probe being labeled by nick translation according to the manufacturer's instructions (BRL, Gaithersburg, MD). The probe was isolated from a cDNA library derived from PMA-stimulated EL4.E1 cells. The library was screened with two oligonucleotides lying near the 5'- and 3'-termini of the coding region. The probe is essentially identical with the mouse IL-2-receptor cDNA described by Miller et al. (24).

Binding of radiolabeled IL-2. The ^{125}I-labeled IL-2 binding assay was based on the method described by Lowenthal et al. (25). Between 5 and 10 × 10^5 MTL2.8.2 cells were incubated in a 96-well round-bottomed microtiter plate with serial dilutions of ^{125}I-labeled IL-2 (36 μCi/μg; NEN Research Products) in a final volume of 0.1 ml of RHFM containing 0.02% sodium azide. After incubation at 4°C for 2 h with constant shaking on a plate shaker, cell-bound and free radioactivity were separated by centrifuging 80 μl of the cell mixture through silicone oil. The average number of binding sites per cell and the binding affinities were determined by weighted least squares regression analysis using the PC version of LIGAND (26). Nonspecific binding was treated as a separate parameter in the regression analysis.

DISCUSSION

Effect of IL-4 on primary MLR cells. It has been reported that in both murine (11) and human cell systems (12), the addition of IL-4 at the beginning of a primary MLR culture increases the level of cytolytic activity generated. To confirm this effect, we added either 4 ng/ml of recombinant mouse IL-4 (32 ED_{50} U/ml), or 10 or 100 U/ml of recombinant human IL-2 to MLR cultures containing CBA/J spleen responder cells and irradiated Balb/c splenic stimulator cells. After 5 days in culture, cytotoxic activity was assessed against P815 target cells. Compared with the control MLR, IL-4 increased the generation of cytotoxic activity by approximately fourfold on the basis of recovered cells (Table I). The total lytic activity generated per culture was increased to a lesser degree, ~2.5-fold. IL-2 had less of an effect, with 10 U/ml actually slightly diminishing the resulting CTL activity, and 100 U/ml increasing it somewhat.

When an antibody that neutralizes IL-4 (11B1) was added to the 5-day MLR, the LU/10^6 cells value was inhibited to about one-half of the control and the total lytic activity to about one-third. These results suggest that IL-4 is normally produced during the MLR and that it enhances the generation of cytotoxic cells.

The simulatory effect of IL-4 on the generation of cytolytic activity may be the result of a specific enhancement of the proliferation of cytotoxic cells or to differentiation of precytotoxic cells or both. To assess the response of MLR-generated CTL to this lymphokine more directly, cells were recovered from MLR cultures on day 5 and recultured in IL-4 or IL-2, or in combinations of the two lymphokines. Proliferation was assessed by the uptake of [^3H]thymidine, with the results shown in Fig. 1. IL-4 by itself hardly stimulated the proliferation of the MLR cells at all, the maximum response being <10% as great as that obtained with IL-2. The highest level of IL-4 used in this experiment was tenfold greater (40 ng/ml, corresponding to 320 ED_{50} U/ml) than that which increased the primary CTL response fourfold in Table I.

When MLR cells were cultured with a combination of IL-2 and IL-4, however, a synergistic effect on short-term proliferation was seen. As shown in Fig. 2, incubation with 13.3 ng/ml of IL-4 significantly increased the response to low levels of IL-2. For example, at 1.23 U/ml of IL-2 and 13.3 ng/ml of IL-4, there was a response of ~5350 cpm compared with responses with either lymphokine alone of 1430 and 430 cpm for IL-4 and IL-2, respectively. There was a synergistic effect which increased the response by ~3600 cpm above the additive

TABLE I

Effect of IL-4 on the CTL response in a primary MLR[a]

Conditions of MLR Culture	LU/Culture	LU/10^6 Cells
No added lymphokine	91	23.8
IL-2 (10 U/ml)	67	17
IL-2 (100 U/ml)	167	31.3
IL-4 (4 ng/ml)	250	91
20 U/ml IL-2[b]	91	33
+ α-IL-4	29	14.3

[a] MLR cultures were established using 1×10^6 splenic cells/ml each of CBA/J responder cells and irradiated Balb/C stimulator cells. The two parts of the table represent two separate experiments. IL-2 or IL-4 were added as indicated at the outset of culture. After 5 days of incubation, viable cells recovered by density gradient centrifugation were washed and counted, and a proportion of the culture was set up in an cytotoxicity assay using P815 target cells. The results are shown as total LU/culture and as LU/million cells recovered on day 5.

[b] 20 U/ml of IL-2 was present at $t = 0$, with or without α-IL-4 monoclonal antibody 11B11 at 1:500 dilution.

[4] Abbreviation used in this paper: MTT-3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide.

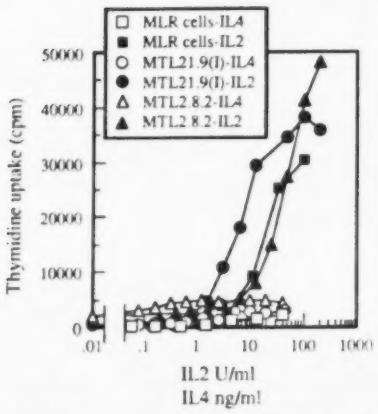

Figure 1. IL-4 does not stimulate the proliferation of primary MLR cells or CTL cell lines. Primary MLR cells were obtained from 5-day cultures of CBA/J splenic responder cells and irradiated Balb/c splenic stimulator cells, as described in Table I, with no added cytokines. MTL21.9(I) cells were reactivated with IL-2 and antigen as described in *Materials and Methods*, and nonviable cells were removed by density gradient centrifugation. Cells were incubated for 48 h with the indicated concentrations of cytokines. Each determination was set up in triplicate and proliferation was assessed by the uptake of [^{3}H]thymidine. MTL21.9(I) cells are dependent on both antigen and IL-2 for continued growth, whereas MTL2 8.2 cells are antigen-independent, but IL-2-dependent.

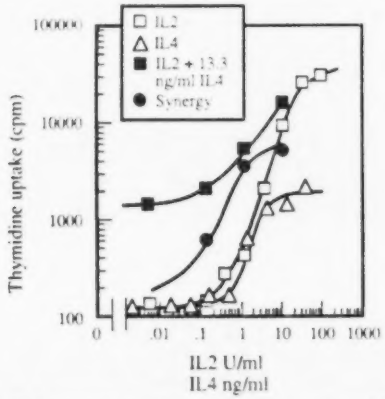

Figure 2. IL-4 synergizes with IL-2 to increase the proliferative response of primary MLR cells. Primary MLR cells, generated in the absence of exogenous cytokines, were incubated with IL-2 alone, IL-4 alone, or 13 ng/ml of IL-4 and varying doses of IL-2 for 48 h. Proliferation was assessed by thymidine incorporation. The curve designated "Synergy" indicates the response that is not accounted for by the sum of the responses obtained with the given levels of IL-4 and IL-2.

effects of the two lymphokines alone. As IL-2 levels increased, the magnitude of the synergistic effect reached a maximum, and the maximal response obtained with IL-4 present was the same as that obtained with IL-2 alone. These observations suggest that IL-4 may in part enhance the recovery of CTL from a primary MLR by selectively augmenting the proliferative response of the activated CTL at low levels of IL-2.

Effect of IL-4 on a IL-2 and antigen-dependent CTL cell line. Cells recovered from the MLR represent a mixed population of cells, and it is therefore not possible to determine whether IL-4 is acting directly on CTL or indirectly through another type of cell. To examine the effects of IL-4 on CTL more directly, we used a cloned line that is both antigen- and IL-2-dependent for growth and cytotoxicity (type 1 CTL). The cloned CTL line MTL21.9(I) was allowed to become quiescent by incubation in IL-2 alone for ~3 wk, and then reactivated by exposure to antigen in the presence of IL-2. Under these circumstances, type 1 cells proliferate for 7–10 days before a gradual decrease in proliferative rate and cytotoxicity. The viability of quiescent type 1 cells can be maintained by IL-2 alone (18).

In contrast to the ability of IL-2 to maintain quiescent MTL21.9(I) cells, IL-4 did not support their survival. After their removal from IL-2, incubation of quiescent cells in concentrations of IL-4 ranging from 1 to 40 ng/ml failed to prevent a decrease in the numbers of viable cells of >90% by 24 h (data not shown). As shown in Fig. 1, reactivated MTL21.9(I) cells proliferated very poorly in response to IL-4 even in the short term. At 40 ng/ml of IL-4, the uptake of [^{3}H]thymidine into DNA after 48 h of culture was ~1% of that attained with 100 U/ml (~10 ng/ml) of recombinant IL-2. Activated MTL21.9(I) cells were viable in the presence of IL-4 for ~5 days before significant cell death.

To investigate whether IL-4 could replace IL-2 or augment it in the reactivation of the cytotoxic activity of type 1 cells, quiescent MTL21.9(I) cells were incubated with antigen and IL-2 and/or IL-4 as shown in Table II. IL-4 alone, or in combination with a suboptimal concentration of IL-2, would not support the reactivation of quiescent type 1 cells, as assessed by increased cytotoxicity.

Experiments were next done to determine if IL-4 affected the response of the cloned CTL line to IL-2. MTL21.9(I) cells were reactivated, washed, and cultured with IL-2, IL-4, or both in combination for 24 or 48 h. Proliferation was assessed by incorporation of [^{3}H]thymidine, as shown in Fig. 3. The addition of IL-4 dramatically increased the proliferation of type 1 cells at low levels of IL-2. For example, at an IL-2 concentration of 0.4 U/ml, the addition of 2 ng/ml of IL-4 augmented the response by about tenfold. The synergistic effect could be detected within 24 h by an increase in both thymidine incorporation (Fig. 3) and cell number (data not shown). These data indicate that IL-4 cannot replace IL-2 in stimulating the growth and reactivation of the CTL clone, but that it will augment cellular proliferation induced by suboptimal levels of IL-2.

Effect of IL-4 on an IL-2-dependent antigen-inde-

TABLE II

IL-4 does not support the reactivation of type 1 CTL[a]

Conditions	Specific ^{51}Cr Release (%)	
	Experiment 1	Experiment 2
Antigen	0	0
20 U/ml IL-2	15.8	3.2
20 U/ml IL-2 + antigen	41.0	34.7
2 U/ml IL-2	ND[b]	1.2
2 U/ml IL-2 + antigen	13.4	6.4
IL-4	3.2	3.9
IL-4 + antigen	3.3	0
2 U/ml IL-2 + IL-4 + antigen	6.1	7.8

[a] MTL 21.9(I) cells were cultured with IL-2 alone for 1 mo after the last reactivation with antigen. These quiescent cells were then incubated with lymphokines and/or antigen as indicated. After 72 hr, the cultures were harvested, washed, and a proportion of each was set up in a cytotoxicity assay with S194 tumor cells as alloantigenic targets. In Experiments 1 and 2, the IL-4 concentrations were 5 ng/ml and 4 ng/ml, and the results represent 2.5 and 5% of the cultures, respectively. Results are expressed as the mean of triplicate samples. The SD was consistently <10% of the mean.

[b] ND, not done.

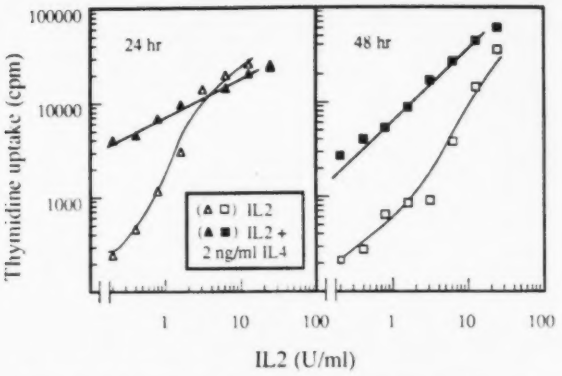

Figure 3. IL-4 synergizes with IL-2 to increase the proliferative response of an activated type I CTL line. Quiescent MTL21.9(l) cells were reactivated by incubation with 20 U/ml of IL-2 and irradiated Balb/c spleen cells for 72 h. Nonviable cells were removed by density gradient centrifugation and the viable cells were incubated with IL-2 or IL-2 plus 2 ng/ml of IL-4 for 24 or 48 h. Proliferation was measured by the uptake of tritiated thymidine into DNA. The responses in the absence of IL-2 were as follows: at 24 h, the control (no IL-4) was 390, plus IL-4, 2980; at 48 h, the respective values were 285 and 1187.

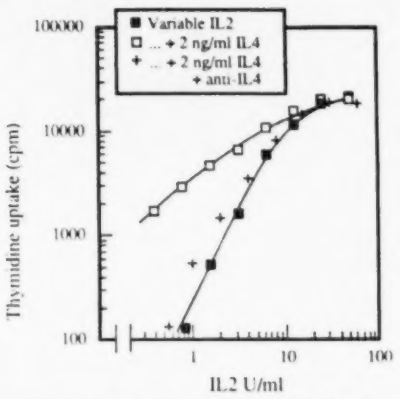

Figure 4. The synergistic effect of IL-4 is blocked by anti-IL-4 antibody. MTL2.8.2 cells were plated in triplicate at 1×10^4 cells/well, with varying concentrations of IL-2, with or without 2 ng/ml of IL-4. The curve indicated represents cultures in which a 1:500 dilution of rat anti-IL-4 antibody (11B11) was present. Incorporation of thymidine into DNA was measured after 48 h.

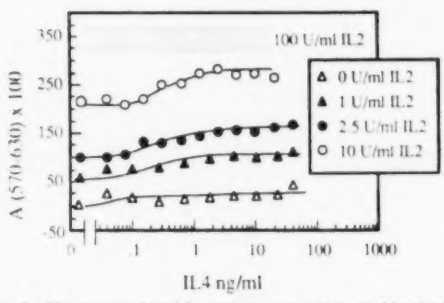

Figure 5. The maximal proliferative response is limited by the level of IL-2 not IL-4. MTL2.8.2 cells were plated in triplicate at 5×10^3 cells/well in a 96-well microtiter plate in a total volume of 0.1 ml with the indicated fixed concentrations of IL-2 and the variable levels of IL-4 indicated. After 48 h, proliferation was measured in the MTT assay. The background is subtracted for each curve. A complete titration curve with IL-2 alone was also carried out and yielded the maximal response shown for 100 U/ml (shaded bar).

pendent, CTL cell line. Type I CTL, which are antigen-specific and -dependent, can be converted to a form that displays a broader specificity of killing, that we have called type II cells (18). This conversion is attained by incubating cells with antigen or PMA and high concentration of IL-2 (100 U/ml or greater), or by exposing them to a partially purified cytokine preparation derived from induced EL4.E1 cells (18). Because the latter preparations probably contain IL-4, we wished to determine if IL-4 is a "conversion factor" for type I cells. Therefore we tested the ability of recombinant IL-4 in combination with antigen or PMA and IL-2 to convert the antigen-dependent line MTL21.9(l) to antigen-independent growth. Using a concentration of IL-2 which is suboptimal for conversion (50 U/ml or less), and IL-4 at concentrations up to 100 ng/ml with antigen or PMA, no evidence of stable conversion was seen. It was noted, however, that during the initial 72 h of culture the cells did take on an appearance similar to that of type II cells, in that they became adherent, larger, and more granular in appearance. This type II phenotypic appearance could not be maintained, however, even when the supernatant was replaced with media containing antigen or PMA and the appropriate cytokines. These results suggest that IL-4 is not the factor that converts type I to type II cells (18).

As shown in Fig. 1, MTL2.8.2 cells, a representative type II line, also does not proliferate significantly in response to IL-4 alone. Similarly to primary MLR cells and type I CTL, however, when MTL2.8.2 cells were cultured with IL-2 plus IL-4 an enhancement of proliferation was seen at low levels of IL-2 (Fig. 4). The specificity of the effect for IL-4 is shown by its abrogation by the anti-IL-4 monoclonal antibody 11B11.

These results indicate that IL-4 cannot replace IL-2 as a maintenance or growth factor for the type I or II CTL cloned lines studied here, but that it acts as a potent helper factor to augment IL-2-dependent proliferation. Additional experiments were done to determine what effect increasing IL-4 levels could have in the presence of suboptimal concentrations of IL-2. For these experiments, we used MTL2.8.2 cells and the MTT colorimetric assay. As shown in Fig. 5, the response of the cells to various submaximal levels of IL-2 was increased to only a limited extent by IL-4 and did not approach the maximal response obtained with saturating IL-2 levels. In other words, the maximal level of proliferation was determined by the concentration of IL-2, not IL-4. These data support the notion that IL-4 is not a growth factor for these CTL lines, but acts synergistically to increase their response to suboptimal levels of IL-2.

Effect of IL-4 on IL-2 receptor mRNA and IL-2 binding to MTL2.8.2 cells. In principle, IL-4 could alter the CTL responsiveness to IL-2 by modulating the number or affinity of IL-2 receptors. Total cytoplasmic RNA was isolated from CTL clones that had been incubated for various times with IL-2, IL-4, or combinations of the two. The results of one experiment with MTL2.8.2 are shown in Fig. 6, in which mRNA levels were compared between equivalent cell numbers. Control cells were cultured in a low level of IL-2 (1 U/ml) instead of medium alone, so that they would remain viable. At 1.5 h the levels of mRNA for the 55 kDa IL-2 receptor chain were similar for cells incubated with any concentrations of IL-2 or IL-4 alone. There was an increase in cells that were cultured

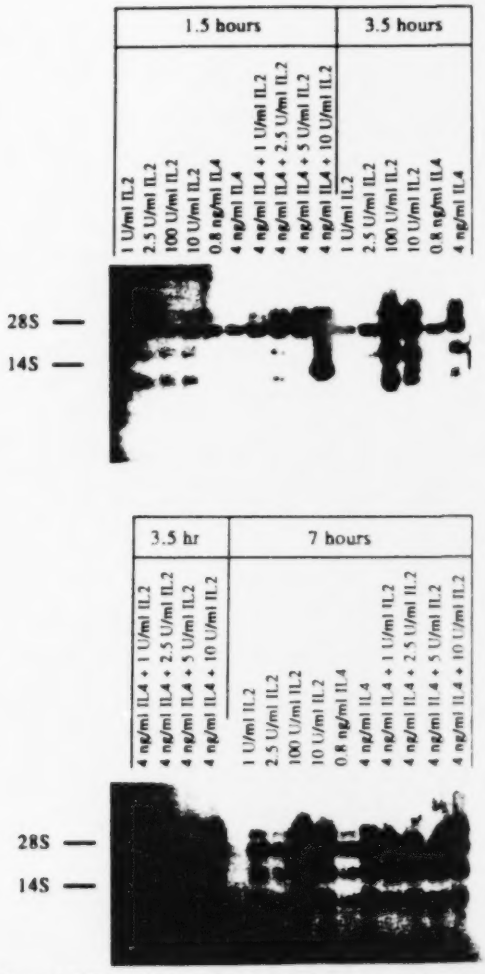

Figure 6. Effects of IL-2 and IL-4 on the level of the p55 IL-2 receptor chain mRNA. MTL2.8.2 cells were incubated with the indicated concentrations of cytokines. At 1.5, 3.5, and 7 h, cells were washed and RNA isolated for Northern analysis. RNA from 2×10^6 cells was loaded per track. The conditions of culture were as indicated above each track. The sizes of the various IL-2 transcripts shown here are essentially the same as those described earlier for the mouse p55 mRNA (23).

with IL-2 plus IL-4, however. With 4 ng/ml of IL-4 added to IL-2 concentrations below 5 U/ml, the increase was similar to the general increase in RNA levels, as suggested by the ethidium bromide staining of ribosomal RNA (data not shown). However, incubation with 10 U/ml of IL-2 and IL-4 produced a specific increase in IL-2 mRNA. By 3.5 h, however, the receptor mRNA under these conditions was not appreciably different from that seen with 10 U/ml of IL-2 alone. By this time, and also at 7 h, IL-4 alone did generate an increase in receptor mRNA, but we cannot exclude the possibility that this increase depended on residual IL-2 bound to the cells. In any case, the increase was no greater than seen with IL-2 alone. In other experiments using type 1 cells the pattern was similar, with a peak of transiently enhanced expression detectable within 1–4 h after initiation of culture. The response was specific for IL-4, as it was inhibited by the anti-IL-4 antibody 11B11. Although these results indicated that a transient rise in IL-2 receptor gene expression was induced by IL-4 plus IL-2, they did not prove a functional relationship.

The effects of IL-2 and IL-4 on functional IL-2 receptors, as distinct from the levels of mRNA for one chain of the receptor, were therefore assessed. To quantitate the number of IL-2 receptors per cell and their affinity for IL-2, MTL2.8.2 cells were incubated at 37°C for 3 h, either in culture medium alone, 1 U/ml of IL-2, 8 ng/ml of IL-4, or in IL-2 plus IL-4. The binding of ^{125}-labeled IL-2 was then carried out. As shown in Table III, no significant difference ($P > 0.5$) in receptor number of affinity could be detected between the four treatments. Although these results do not exclude the possibility that IL-4 induces a transient increase in IL-2 receptor levels, as it does in the corresponding mRNA, it does not appear to enhance the IL-2-dependent proliferation by this mechanism, because no significant increase in receptor function was detectable by 3 h after stimulation, yet synergistically-enhanced proliferation was observed (Fig. 3).

DISCUSSION

In this study we have examined the effects of IL-4 on the proliferation of CTL including primary MLR cells and two phenotypically distinct CTL cell lines. IL-4 was not significantly mitogenic on its own. However, it was synergistic with low levels of IL-2, increasing the incorporation of thymidine into DNA by tenfold or more. This enhancement was blocked by monoclonal anti-IL-4 antibody. Spits and coworkers (7) found that IL-4 in conjunction with IL-2 could increase the proliferation of a human CD4$^+$ cell line. This effect was only apparent after the cells had been maintained in culture for longer than 4 days. It is possible that this synergy was mediated by an additional factor secreted from the helper cell clone. Umadome et al. (27) recently found that IL-4 stimulated growth of human T lymphocytic leukemia cells, as did IL-2, and that IL-4 and IL-2 synergized to generate much higher levels of proliferation than either agent alone. This result differs from ours in that IL-2 alone did not produce the maximal proliferative activity, being no more effective than IL-4, whereas in our systems, the maximal proliferative response was achieved with saturating IL-2 levels alone, and IL-4 was barely mitogenic.

Several lines of evidence suggest that the increased proliferation induced by IL-4 in the present study was not the result of an autocrine release of IL-2. Although many cloned T lymphocyte cell lines have the capability for both helper and cytotoxic function (28), we have not seen any evidence for lymphokine production by these CTL lines, and there was no detectable cytoplasmic IL-2 mRNA in cells cultured with IL-2, IL-4, or IL-2 plus IL-4 (data not shown). The synergistic effect was not modified by the addition of cyclosporine, an agent known to block the transcription of IL-2 and other cytokine genes (23, 29), further suggesting that autocrine production of a soluble mediator was not part of the IL-4-induced effect.

Although it has been reported that T cells of both the helper and cytolytic phenotype proliferate in response to IL-4, it is becoming increasingly clear that the mechanism by which the interaction of the cytokine with its receptor translates into a functional response is distinct from that of IL-2. It has been shown that IL-4, when added exogenously to a mouse (11) or human (12, 13)

TABLE III

Effects of IL-2 and IL-4 on IL-2 receptors[a]

Treatment	High Affinity Receptors		Low Affinity Receptors	
	K_D (pM)	Sites/cell	K_D (nM)	Sites/cell
Medium	46 ± 33	1,228 ± 663	5.3 ± 2.0	49,800 ± 12,500
IL-2	62 ± 38	1,068 ± 534	6.3 ± 1.8	53,800 ± 10,800
IL-4	61 ± 60	1,003 ± 862	3.7 ± 1.3	40,100 ± 7,600
IL-2 + IL-4	86 ± 45	1,758 ± 756	9.5 ± 3.9	67,200 ± 20,200
Combined regression	63 ± 22	1,325 ± 371	5.7 ± 1.0	53,000 ± 5,800

[a] MTL2.8.2 cells (2×10^6/ml) were incubated for 3 hr at 37°C in medium, 1 U/ml of IL-2, 8 ng/ml of IL-4, or IL-2 plus IL-4. They were then washed and resuspended at 4°C before exposure to radioiodinated IL-2. Further details of the binding assay and data analysis are described in *Materials and Methods*. The combined regression of the four curves was not significantly different ($P > 0.5$) from regression of the four curves separately.

MLR enhances the recovery of cytolytic activity. IL-4, unlike IL-2, does not induce the development of LAK cells from unprimed human PBL (13) and reduces the level of LAK activity when added simultaneously with IL-2 (12). In a murine system, high levels of IL-4 induced nonspecific lytic activity although the efficacy was less than that of IL-2 (30). It has also been shown that dexamethasone affected the proliferative response of CTLL2 cells to IL-2 differently from the response to IL-4 (31).

In this study, a difference in signaling mechanism between IL-4 and IL-2 is strongly suggested by two kinds of data. In the first place, IL-4 did not maintain or reactivate quiescent type I CTL, as IL-2 did. It has little proliferative effect on activated MTL21.9(I) cells or on the antigen-independent, IL-2-dependent cell line MTL2.8.2. The other relevant type of data showed that, at a fixed, nonsaturating concentration of IL-2, adding IL-4 did not increase the proliferative response beyond the synergistic level (Fig. 5). In other words, the maximal proliferative response was determined by the level of IL-2 not by IL-4. This indicates that, at least in the cells being studied here, IL-4 modulated the responsiveness of the cells to IL-2 by a signaling mechanism that is not identical with the one activated by IL-2 itself.

Another difference between IL-2 and IL-4 in the present study is that only IL-4 significantly increased the lytic activity in the MLR (Table I). The addition of exogenous IL-4 increased cytotoxicity, and inclusion of α-IL-4 antibody 11B11 diminished it. Exogenous Il-2 had a smaller effect. The basis of the IL-4 effect cannot be definitively established. IL-4 could be inducing the selective proliferation of the specific cytotoxic subpopulation of cells, or it could be inducing a differentiation of precursors to effectors at a higher rate. It should be kept in mind that the normal MLR occurs in the continuous presence of IL-2. The measured IL-2 level is continuously ~10 U/ml over a 5-day period of the MLR (32). At 10 U/ml, however, cells recovered from a 5-day MLR exhibit suboptimal proliferation (Fig. 1) and are subject to synergistic stimulation by IL-4. Thus the effect of IL-4 on proliferation documented here could account for a strong stimulatory effect on the cytotoxicity generated in an MLR. It might seem puzzling that addition of exogenous IL-2 did not have as much of a stimulatory effect on the CTL generated as IL-4 did. This is probably because exogenous IL-2 is rapidly consumed in such cultures, and it is difficult to greatly influence the steady state level. For example, addition of 100 U/ml of exogenous IL-2 to an MLR was found to have no effect on the level of IL-2 in the culture by day 5. There appears to be a regulatory mechanism that ensures that the steady-state IL-2 level under these conditions never rises much above 10 U/ml. Thus the cultures would be sensitive to the synergistic effect of IL-4 documented here.

The molecular mechanism by which IL-4 modulates the IL-2-dependent proliferative response is unknown, but it does not appear to be mediated by an alteration in the function of the IL-2 receptor. Although a transient increase in the mRNA for the 55 kDa IL-2 receptor chain was detected, there was no significant change in the binding of radiolabeled IL-2 because of pretreatment of MTL2.8.2 cells with IL-4 or IL-2 plus IL-4. It is possible that IL-4 augments the IL-2-dependent response by altering the sensitivity of the cell to the interaction of IL-2 with its receptor, resulting in entry into the cell cycle at a lower IL-2 binding density than would otherwise be required.

The results of this study complement observations of Carding and Bottomly (15) who used fractionated thymocytes. They demonstrated an enhancement of proliferation of Lyt-2$^+$ thymocytes incubated with IL-4 in the presence of IL-2 and a suboptimal concentration of PMA. Taken together, the studies suggest a role for IL-4 in the expansion of CTL precursors and committed CTL.

It has been proposed that at least two distinct subsets of T helper cells, T_{H1} and T_{H2}, can be distinguished by their pattern of cytokine release (33). It is possible that IL-4 secretion from T_{H2} cells plays a role in the CTL response by augmenting the IL-2-dependent growth of antigen-activated CTL, at a time when IL-2 secretion from T_{H1} cells is limiting. Such a mechanism would lend a greater flexibility to cellular responses in vivo. The synergistic effects documented here also have implications for attempts to use lymphokines for immunotherapy, as they suggest that lower levels of a given effector (e.g., IL-2) can be used if a second (e.g., IL-4) is presented simultaneously. This could well lead to increased efficacy at doses which produce fewer side effects.

Acknowledgment. We are grateful to Dr. Kim Bottomly (Yale University) for the anti-IL-4 antibody 11B11.

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0022-1767/90/1443-0816$02.00/0
THE JOURNAL OF IMMUNOLOGY
Copyright © 1990 by The American Association of Immunologists
Vol. 144, 816–823, No. 3, February 1, 1990
Printed in U.S.A.

DEVELOPMENT OF PRECYTOTOXIC T CELLS IN CYCLOSPORINE-SUPPRESSED MIXED LYMPHOCYTE REACTIONS[1]

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Cyclosporine (CsA) blocked the generation of cytolytic activity in a primary MLR of mouse spleen cells. As expected from the known mechanism of action of this drug, it also blocked the accumulation of IL-2 during the MLR. Addition of human rIL-2 did not overcome the inhibition of CTL generation, even when it was added daily to keep its level similar to that produced in a normal MLR. Daily addition was necessary, because the CsA-inhibited MLR consumed IL-2, either by utilization or degradation. The outcome of a 5-day MLR in the presence of CsA (CsA-MLR) depended on whether or not IL-2 was continuously present. In the presence of IL-2, there was no generation of CTL activity, probably because such cultures contained IL-2-dependent suppressive elements described previously. However, when day 5 CsA-MLR cells generated in the absence of IL-2 were washed and recultured with human rIL-2, there was a burst of CTL activity, with a more than 50-fold increase in alloantigen-specific cytotoxicity within 24 to 48 h. This increase is not explainable simply by the proliferation of existing effector CTL. The noncytotoxic cells produced in an MLR in the presence of CsA, and which can be rapidly activated to cytotoxic effector cells by IL-2, are termed "precursor-effector CTL" (peCTL). They could be detected by day 3 of a primary CsA-MLR culture. Their conversion to effector CTL by IL-2 was not inhibited by CsA. Exposure of peCTL to IL-4 also generated CTL activity, to a somewhat lesser degree than IL-2, but the IL-4-induced activation was inhibited by CsA, suggesting that it depended on the induction of another CsA-sensitive lymphokine. The intracellular levels of mRNA encoding the CTL-specific serine esterases CCP1 and CCP2 (granzymes B and C, respectively) increased rapidly during the IL-2-driven conversion of peCTL to effector CTL. This study demonstrates that in the presence of CsA precursors for CTL can accumulate, and that these can be rapidly converted to cytotoxic effector cells by IL-2.

CsA[3] is a clinically useful immunosuppressive agent because of its relatively specific ability to inhibit immune reactions to tissue allografts, but the mechanisms of its actions in complex systems are only partly understood. The in vitro model of alloreaction provided by the MLR is often used to study the effects of CsA. Its inhibitory effect on the generation of CTL in an MLR is in part attributable to its ability to block the release of IL-2 (1–3). CsA may also block the maturation of CTL by inhibiting the induction of several other cytokines, including IFN-γ (4, 5) and CSF (6, 7).

IL-2 appears to overcome some of the inhibitory effects of CsA in the MLR. In CsA-treated MLR cultures of human PBL, proliferation was largely restored by IL-2-containing lymphokine preparations, but the generation of CTL was not (8). Analogous studies with mouse cells showed that rhIL2 did not overcome the inhibitory effect of CsA on the generation of CTL in this system either (9). Particularly revealing is the observation that, in limiting dilution MLR cultures, CsA blocked the production of CTL, even in the presence of exogenous IL-2 (10–12). And in cloned lines of Ag-dependent CTL, reactivation by Ag was inhibited by CsA, although delivery of a required "maintenance" signal by IL-2 was not (13).

These lines of evidence and others (14) suggest that it is Ag-mediated signaling in T lymphocytes which is particularly sensitive to CsA. Other components of the immune response are also potential targets, however. CsA has been reported to reduce the level of IL-2R expressed in mixed cell populations such as human PBL (15). There is disagreement about the interpretation of this finding, however (16), and it is not usually observed in cloned T cell lines (7). When it does occur, it may be secondary to a CsA-mediated inhibition of IL-2 production, because IL-2 is known to induce an increase in its own receptors (17).

Another aspect of the inhibition of the MLR by CsA is the induction of suppressive cells. Suppression was induced by CsA in guinea pig MLR cultures (18), through the generation of radiosensitive Ts cells. These cells secreted a factor that could partially suppress the proliferation of MLR cells. In MLR cultures of mouse spleen cells, CsA inhibited both the generation of CTL and Ts cells. The addition of exogenous IL-2 to the cultures restored the latter, but not the former (9).

The activation of differentiated, quiescent pCTL to become CTL is a complex process that involves interactions

Received for publication July 19, 1989.
Accepted for publication October 31, 1989.
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[1] This work was supported by grants from the National Cancer Institute and the Medical Research Council of Canada. C. L. M. and C. D. H. are supported by studentships from the Alberta Heritage Foundation for Medical Research.
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[3] Abbreviations used in this paper: CsA, cyclosporine; hIL-2, human IL-2; pCTL, precursor CTL; peCTL, precursor-effector CTL.

with ancillary cells and lymphokines. Potentially, interference with any of several steps can either abrogate the response, interrupt it at an intermediate stage, or inhibit it by producing suppression. The conversion of pCTL to effector CTL can be broken down into discrete steps. For example, it is interrupted at an intermediate, activated stage by the inhibitor pyriiamine, which blocks the conversion of activated precursors to effector function (19). Similarly, ornithine, arginine, or putrescine blocked the generation of CTL activity without affecting IL-2 or IL-3 production, or proliferation, and cytotoxic activity could be recovered if the agents were removed from culture (20). Along different lines, activated pCTL isolated 12 h after exposure to Ag could be converted to effector CTL by exposure either to Ag plus feeder cells, or to lymphokine preparations enriched for IL-2 (21, 22).

In studying the effects of CsA on the generation of CTL from pCTL, we observed that even in the presence of exogenously added IL-2, CsA blocked the generation of CTL at an activated, intermediate stage, which we refer to as peCTL. We describe the conversion of precursor CTL to peCTL, and the requirements for converting these to effector CTL.

MATERIALS AND METHODS

Materials. Powdered CsA (Sandoz Canada Inc., Dorval, Quebec, Canada) was dissolved in DMSO at 1 mg/ml and diluted in culture medium (RHFM). Aphidicolin was obtained from Sigma Chemical Co. (St. Louis, MO). The basic culture medium RHFM consisted of RPMI 1640 (GIBCO, Grand Island, NY), 10% (v/v) FBS (GIBCO), 10^{-4} M 2-ME, and 10 mM HEPES buffer (Sigma).

Cytokines and antibodies. rhIL-2 was secreted from yeast cells transformed with an expression vector containing a synthetic hIL-2 cDNA (23). Purified mouse rIL-4 was provided by the Immunex Corp. (Seattle, WA). Approximately 0.125 ng/ml of this purified material stimulates half-maximal proliferation of the HT-2 cell line. The rat, anti-IL4 mAb 11B11 (24) was kindly provided by Dr. Kim Bottomly (Yale University, New Haven, CT).

Mice and cell lines. CBA/J ($H\text{-}2^k$) and BALB/c ($H\text{-}2^d$) mice were maintained in the Medical Sciences animal facility, University of Alberta, and were used from 10 to 16 wk of age. P815 ($H\text{-}2^d$), S194 ($H\text{-}2^d$), EL4 ($H\text{-}2^b$), and RI ($H\text{-}2^k$) tumor cell lines were maintained in continuous culture in RHFM.

Primary MLR cultures. Spleen cells were obtained aseptically by pressing the spleen through a wire mesh into RHFM. Responder cells (1 to 2 × 10^6/ml) were cocultured with equal numbers of allogeneic stimulator cells (1500 rad from a ^{137}Cs source) in RHFM with the indicated concentrations of CsA and lymphokines in a final volume of 4 ml (Costar 6-well cluster, Costar, Cambridge, MA) or 25 ml (Costar 75-cm^2 tissue culture flask). The cultures were incubated at 37°C in 5% CO_2 and 90% relative humidity.

Reculture. Cells from the primary MLR cultures were harvested, washed in RHFM, and then recultured with cytokines at a cell density of 2 to 5 × 10^5 cells/ml for 24 or 48 h. In some experiments, viable cells were isolated by gradient density centrifugation.

Cytotoxicity assays. Cells were incubated with 10^4 target cells labeled with $Na^{51}CrO_4$ (New England Nuclear, Boston, MA) in a round-bottom microtiter plate (final volume of 200 μl). After 4 h at 37°C, 100 μl of supernatant was removed from each well for counting. Specific lysis was calculated as:

$$\% \text{ lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100$$

Spontaneous release was obtained by incubating ^{51}Cr-labeled targets alone, and total release from target cells incubated with 1% Zapisoton lytic agent (Coulter Electronics of Canada, Ltd., Mississauga, Ontario).

Assay for IL-2. This was performed as described previously (25) using the IL-2-dependent cell line MTL2.8.2, and monitoring the reduction of the tetrazolium dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (26). CsA has no effect on this assay. IL-2 activity is expressed relative to a reference standard from Biological Resources Branch, NCI-FCRF (Frederick, MD). Half-maximal stimulation of T cell lines used in this work occurred between 1 to 5 U/ml IL-2.

Proliferation assay. Cells were cultured in triplicate in a 0.2 ml final volume in 96-well flat-bottom microtiter trays (Costar) with 0.25 μCi/well [^{3}H]TdR (2Ci/mmol, NEN, Boston, MA) for 6 h. Cultures were harvested on a Skatron harvester (Lier, Norway) and the radioactivity incorporated was determined by β-scintillation counting.

RNA isolation and Northern analysis. Cells were homogenized in guanidinium thiocyanate and RNA was recovered by sedimentation through cesium chloride (27). RNA pellets were resuspended in 0.3 M sodium acetate, ethanol precipitated and washed with 70% ethanol. Denatured RNA was separated on a 1% agarose-formaldehyde gel and transferred to nylon membrane (Amersham Corp., Arlington Heights, IL). The blots were probed with nick-translated ^{32}P-labeled cDNA probes B10 and C11 (28), which encode the CTL-derived serine protease genes CCPI (granzyme B) and CCPII (granzyme C), washed, and the bands visualized by autoradiography.

RESULTS

IL-2 added early, or continuously, does not overcome inhibitory effect of CsA on generation of CTL in primary MLR. CsA inhibits the generation of cytotoxic cells in mouse or human MLR cultures (1, 8, 9, 18), but the precise relationship of inhibited IL-2 release to this effect has not been delineated. In particular, in past studies it has not been determined whether or not exogenously added IL-2 persists throughout CsA-inhibited cultures, or whether it is rapidly used up. We therefore added IL-2 and also assayed its levels in such cultures. A primary MLR was set up in which varying combinations of CsA and rhIL-2 were added at the onset of culture. On day 5 the specific cytotoxic activity and the amount of IL-2 in the culture supernatants were determined, with the results shown in Table I. CsA inhibited the generation of CTL, reaching a level of 95% efficiency at 125 ng/ml. The addition of rhIL-2 increased the CTL response somewhat, particularly at the lowest level of CsA (62.5 ng/ml), but did not significantly reverse the inhibition seen at 125 ng/ml or higher levels. Any effects of IL-2 were essentially complete by 20 U/ml, suggesting that the lack of complete restoration was not because insufficient rhIL-2 was added.

As also shown in Table I, no IL-2 was recovered from the cultures on day 5 in the presence of the higher levels of CsA, indicating that CsA was having the expected effect of blocking IL-2 production. However, the addition of 100 U/ml rhIL-2 led to the recovery of more-or-less normal amounts of IL-2 at the end of the 5-day cultures (5 to 10 U/ml), without restoring cytotoxicity, indicating that the lack of the latter was not solely the result of a lack of IL-2.

Curiously, the addition of even relatively massive

TABLE I

Effects of CsA on CTL generation in MLR[a]

IL2 Added (U/ml)	% Lysis (IL-2 Recovered, U/ml)			
	0	20	50	100
CsA (ng/ml)				
0	77 (9.6)	86 (6.4)	86 (8.5)	93 (7.3)
62.5	28 (16.0)	48 (8.8)	55 (9.2)	51 (15.5)
125	4 (4.7)	6 (2.3)	13 (3.2)	14 (4.8)
250	1 (1.3)	0 (0)	4 (0)	10 (3.8)
500	0 (0)	0 (0)	1 (1.3)	6 (5.0)
1000	0 (0)	0 (0)	2 (0)	6 (8.4)

[a] Primary MLR cultures were set up as described in Figure 1 with the indicated concentrations of rhIL-2 and CsA. IL2 levels present in the various cultures on day 5 were quantitated by bioassay as described in *Materials and Methods*. Cytotoxicity was assayed against ^{51}Cr-labeled P815 target cells and is expressed as percent lysis using a 6/1 E/T ratio. In several similar experiments of this type no significant cytotoxicity was observed in the absence of antigenic stimulator cells. The concentration of IL-2 detected on day 5 in the various cultures is given in parentheses.

amounts of rhIL-2 (100 U/ml) in the absence of CsA did not affect the level of IL-2 present on day 5. The cultures appeared to be buffered with respect to IL-2 levels. However, when endogenous IL-2 production was inhibited by CsA, there was no detectable IL-2 present on day 5, even if 50 U/ml were added at the outset. Significant IL-2 on day 5 was found only at the highest level of exogenous IL-2 addition. There was a very significant rate of IL-2 consumption at all levels of CsA, but quite clearly this consumption was not the result of generating effector CTL.

Given the ability of CsA-inhibited MLR cultures to consume even relatively high levels of exogenous IL-2, the only way to simulate normal IL-2 levels was to add it daily throughout a 5-day MLR, as described in Figure 1. To insure that normal levels were being simulated, the IL-2 concentration in culture was assayed on days 1, 3, and 5 (aliquots of the cultures were removed just before the daily addition of IL-2). As shown in Figure 1*A*, the addition of 10 U/ml/day of rhIL-2 did not significantly restore the CTL response in CsA-suppressed cultures, although it did maintain the IL-2 levels at values similar to those present in control cultures (Fig. 1*B*). As implied by the experiment outlined in Table 1, IL-2 added at the onset of CsA-MLR cultures was consumed or inactivated over the culture period. Taken together, these results show that the suppressive effect of CsA on the MLR cultures was not due solely to the inhibition of IL-2 production.

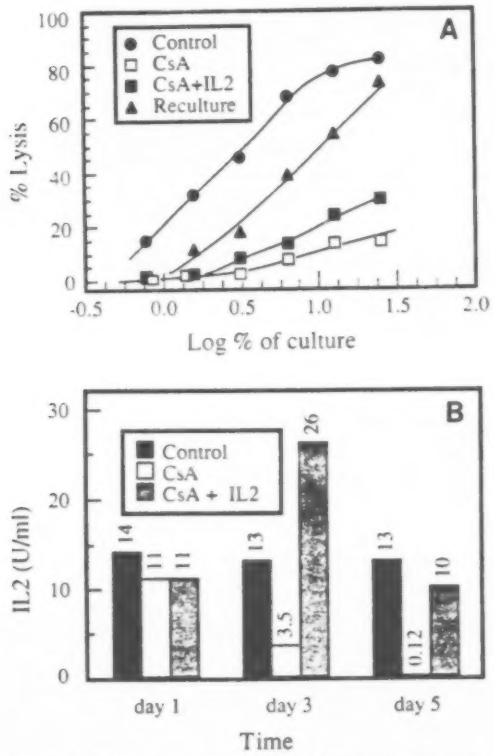

Figure 1. IL-2 does not overcome CsA-mediated suppression of cytotoxicity in a primary MLR. Primary MLR cultures of CBA/J spleen responder cells and irradiated BALB/c spleen stimulator cells (1×10^6/ml each) were initiated in the presence of 20 U/ml rhIL-2. The cultures were treated as follows: control—no further additions; CsA—300 ng/ml CsA on day 0; CsA + IL-2—300 ng/ml CsA on day 0, and 10 U/ml rhIL-2 on each of the next 4 days; reculture—300 ng/ml CsA on day 0, and on day 4 the cells were harvested and washed, and recultured for 24 h in 10 U/ml rhIL-2. *A*, cytotoxicity against ^{51}Cr-labeled S194 tumor cells of MLR cells harvested on day 5. The abscissa is the $\log_{10}$ of the percent of the original cultures that were present in each of the cytotoxicity assays. Thus, a value of 1.0 indicates that 10% of the original culture was taken for that particular chromium release assay. *B*, IL-2 levels. On the days indicated, 0.1 ml of supernatant was removed for IL-2 determination. (Sampling was done before the daily addition of IL2 to the CsA + IL-2 cultures.)

IL-2 and IL-4 rapidly activate latent cytolytic activity in CsA-suppressed MLR cultures. To investigate the rapid activation of CsA-suppressed cells, as illustrated by the effect of reculturing day-4 CsA-MLR cells (Fig. 1*A*), primary MLR cultures were set up with 300 ng/ml CsA and 20 U/ml of rhIL-2. After 5 days the cells were washed and recultured with graded doses of rhIL-2 or 4 ng/ml IL4 for 24 h, at which time specific cytotoxicity and proliferation were measured (Fig. 2*A*). CsA added at the beginning of the MLR resulted in almost total inhibition of cytotoxic activity measured on day 6. But rhIL-2 added to the recultured cells on day 5 led to a rapid, dose-dependent increase in both cytotoxicity and proliferation within 24 h. A plateau in both activities was observed with concentrations of 5 U/ml rhIL-2 or more (Fig. 2*A*). The increase in cytotoxicity was dramatic. Although difficult to evaluate precisely, because of the very low response of the CsA-suppressed cells, it was at least 50-fold, a value too large to be solely explained by the proliferation of CTL present at day 5.

IL-4 increases the recovery of cytolytic activity in primary cultures (29, 30). Also, it has a synergistic effect with IL-2 in promoting the growth of both cloned and uncloned CTL[4]. We therefore wished to determine if IL-4 would stimulate the rapid conversion of peCTL to CTL in CsA-MLR cultures. As shown in Figure 2*B*, IL-4 at 4 ng/ml did support the generation of cytotoxic activity of recultured cells, although to a lesser extent than that obtained with rhIL-2. This level of IL-4 produced plateau values of responses both in primary CTL cultures (29) and in synergistic effects on IL-2-induced proliferation in primary and cloned cell cultures (see footnote 4). No synergistic effect was observed between IL-2 and IL4 (data not shown). The addition of an anti-mouse IL-4 mAb to cells recultured with IL-2 had little effect on the cytotoxicity generated, although it did reduce DNA synthesis, as measured by [^{3}H]TdR uptake (Fig. 2*B*). This suggests that the main effect of endogenously produced IL-4 was on proliferation, and that IL-2 was not inducing cytotoxicity by way of inducing IL-4 production.

Lack of cytotoxic activity in 5-day CsA-inhibited MLR is not due to soluble inhibitor. If the lack of cytotoxic activity in the CsA-inhibited MLR is due to a soluble inhibitor, its effects would be nullified when the cells are recovered, washed, and recultured in IL-2, as in the majority of the experiments described heretofore. However, the lack of such an inhibitor is indicated by the results

[4] C. L. Miller, J. W. L. Hooton, S. Gillis, and V. Paetkau. 1990. IL-4 potentiates the IL-2-dependent proliferation of mouse cytotoxic T cells. *J. Immunol. In press.*

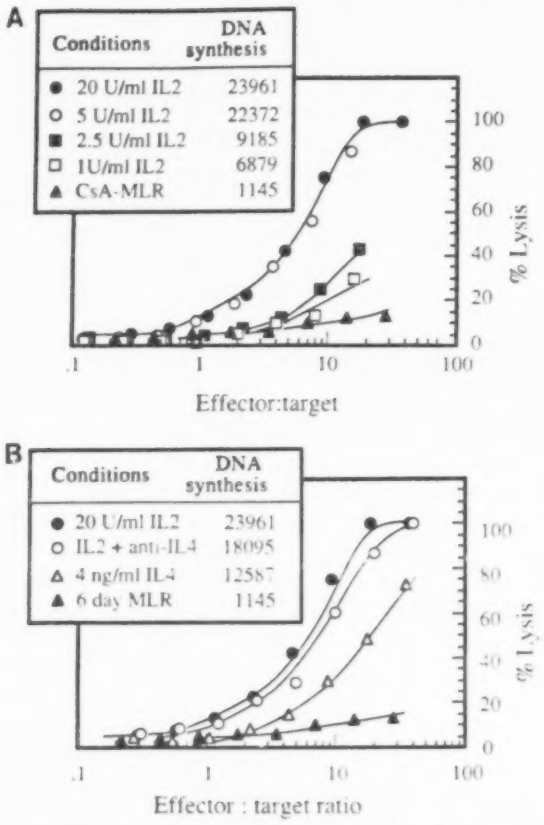

Figure 2. Reculture of primary CsA-suppressed MLR cells in IL-2 leads to a rapid increase in cytotoxic activity. One-way CBA/J α-BALB/c MLR cultures were set up as in Figure 1, in the presence of CsA (300 ng/ml) and rhIL-2 (20 U/ml). On day 5, viable cells were recovered by density gradient centrifugation, washed, and resuspended at 5×10^5/ml in RHFM with varying concentrations of rhIL-2 or 4 ng/ml IL-4. One culture was left untouched until the cytotoxicity assay on day 6 ["CsA-MLR"]. The other cultures were harvested 24 h later and assayed for cytotoxicity against ^{51}Cr-labeled P815 tumor cells. A portion of each culture was also set up in a 6-h proliferation assay, with the results shown for each curve ([^{3}H]TdR incorporation expressed as cpm/10^3 cells). *A*, Effect of IL-2. *B*, Effect of IL4, and of anti-IL-4 on the IL-2 stimulated activation.

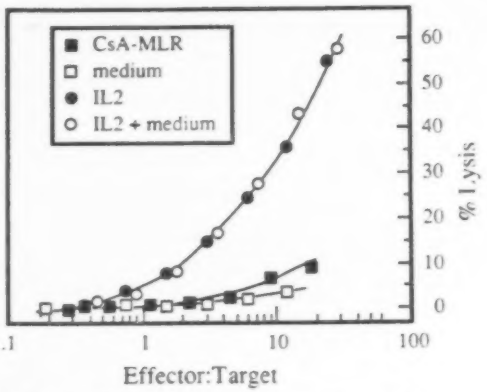

Figure 3. CsA-MLR cultures can be directly activated to cytotoxicity by IL-2. One-way CBA/J α-BALB/c MLR cultures were set up as in Figure 1, and assayed for cytotoxicity on P815 target cells on day 7. CsA was present at 300 ng/ml, and 20 U/ml of rhIL-2 was added on day 0. The culture labeled "CsA-MLR" was not manipulated further. Other cultures were manipulated as follows: medium-medium and CsA to double the original volume on day 5 (1 ml of medium containing 300 ng/ml CsA was added). IL-2—20 U/ml rhIL-2 added on day 5. IL-2 + medium—medium containing 300 ng/ml CsA to double the original volume, and rhIL-2 to a final concentration of 20 U/ml on day 5.

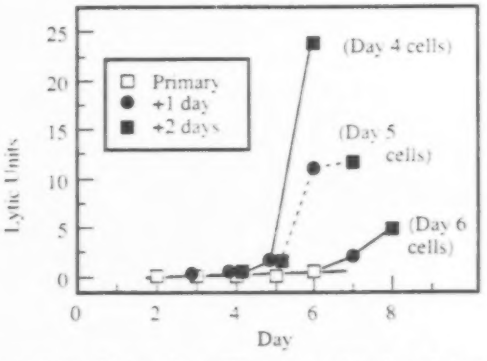

Figure 4. Preeffector CTL (peCTL) emerge relatively early during a CsA-MLR. MLR cultures were set up as in Figure 1, with 300 ng/ml CsA but without exogenous IL-2. On each day of the primary culture, cells were washed and recultured with 20 U/ml rhIL-2 for 1 or 2 days, and cytotoxicity was then assessed on ^{51}Cr-labeled P815 target cells. One LU is defined as the number of cells causing 20% lysis under the conditions of the assay.

in Figure 3, where the addition of IL-2 to 5-day CsA-MLR cultures led to a large increase in cytotoxicity in the next 48 h. Dilution of the medium alone was without effect. Thus, these cells are poised to generate cytotoxicity, and only require IL-2 to do so. This result is notably different from that of Figure 1, where IL-2 was continuously present during the culture, yet no significant CTL activity developed. The significance of this difference is considered in *Discussion*. Further evidence against the existence of a soluble mediator of suppression was the observation that addition of up to 50% by volume of medium from a 5-day CsA-MLR culture to ongoing uninhibited MLR cultures, at days 3, 4, or 5, had no effect on the level of cytotoxicity recovered on day 5 . Nor did such conditioned medium affect the cytotoxicity of cloned CTL cells (data not shown).

Level of peCTL in CsA-suppressed MLR rises and falls rapidly. These results indicate the presence of a population of peCTL in the CsA-MLR, a population that rapidly differentiates from a noncytotoxic phenotype to a cytotoxic one in the presence of IL-2. The rapid generation of cytotoxic activity by the reculture of CsA-suppressed MLR cells with IL-2 is an assay for peCTL. Figure 4 shows an experiment in which cells from a primary CsA-MLR, set up without exogenous IL-2, were taken on each day of the primary culture and recultured in 20 U/ml rhIL-2. Cytotoxicity was determined after 1 and 2 days of the secondary culture. Throughout the primary culture there was a negligible cytotoxic activity, but reculture in rhIL-2 revealed a rise and fall in the level of peCTL in the primary culture, with a peak activity on day 4.

CTL generated by reculture of peCTL are Ag-specific. The exposure of lymphoid cells to high levels of IL-2 produces lymphokine-activated killer cells (31), and exposure of Ag-specific CTL clones to high levels of IL-2 generates Ag-nonspecific cytotoxicity (32, 33). It was therefore important to determine the antigen specificity of the CTL generated by exposing the peCTL to IL-2 or IL-4. As seen in Table II, peCTL that were recultured with

TABLE II

Target specificity of CTL generated from CsA-MLR by reculture in IL-2[a]

Conditions	$LU_{20}/10^6$ Cells on	
	S194 (appropriate target)	EL4 (third party)
Medium	<1[b]	<1[b]
2 U/ml IL-2	2.4	<1[b]
20 U/ml IL-2	43.4	2.5
200 U/ml IL-2	83.3	5.6
4.0 ng/ml IL-4	11.8	<1[b]
6 day CSA-MLR	1.7	<1[b]

[a] A primary MLR was set up as in Figure 1, containing 300 ng/ml CsA and 20 U/ml rhIL-2. On day 5 cells were recovered, washed, and incubated for 24 h with the indicated concentrations of cytokines. (In the last row, the CsA-MLR was left undisturbed for 6 days.) Cytotoxicity was assessed against EL4 (third party) and S194 (specific) target cells. There was no measurable cytotoxicity against (self-haplotype) RI cells (data not shown). One LU_{20} yields 20% specific lysis of the indicated target cells.

[b] Using an E/T ratio of 50/1, specific ^{51}Cr release did not achieve 20% lysis.

IL-4, or with IL-2 at concentrations up to 200 U/ml, were specific for the priming Ag. Similar results were obtained using P815 target cells, which are of the appropriate antigenicity, but that are NK resistant. Thus, the CTL generated by reculture, and their peCTL precursors, were alloantigen-specific CTL.

CsA does not inhibit generation of CTL from peCTL induced by IL-2. Although IL-4 and IL-2 both support the development of cytotoxicity from peCTL, other lymphokines produced during the reculture period may be necessary or contributory. As CsA is known to block the production of a variety of cytokines, its effect was tested during the reculture period. The induction of cytotoxicity by IL-2 was not inhibited by CsA; the cytotoxic activity was identical for CsA-MLR cells recultured in rhIL-2 and in rhIL-2 plus CsA (Fig. 5*A*). However, 5-day CsA-MLR cells that were cultured in fresh medium alone showed some recovery of cytotoxicity in this experiment, and this increase was inhibited by CsA. This quite likely results from the inhibition of the production of IL-2 or another lymphokine.

In contrast to the lack of effect of CsA on IL-2-mediated activation of peCTL, the ability of IL-4 to induce cytotoxicity was completely abolished by CsA (Fig. 5*B*). This indicates that either the effect of IL-4 on the peCTL is directly inhibited by CsA, or that IL-4 acts by inducing another lymphokine, such as IL-2, whose production is inhibited by CsA. Inasmuch as IL-2 itself induces a high level of CTL activity (Fig. 5*A*), it seems quite likely that an obligatory component of the IL-4-induced activation of peCTL is the production of IL-2.

Proliferation appears to be necessary for generation of CTL from peCTL. Cytotoxicity can develop in limiting dilution cultures in the absence of proliferation (30). At the same time, the massive increase in cytotoxicity seen within 24 h in Figure 2*A* indicates that the generation of CTL from the CsA-inhibited peCTL upon exposure to IL-2 is not due simply to proliferation of preexisting CTL. However, these results do not indicate whether or not the developing CTL need to be able to proliferate in order to differentiate into effector cells, as has been suggested (34). Aphidicolin, a potent inhibitor of DNA polymerase α and thus of de novo DNA synthesis (35), was used to examine this possibility. Cells from 5-day CsA-MLR cultures were recultured in the presence of varying concentrations of aphidicolin and either 20 U/ml rhIL-2 or 4 ng/ml IL-4. Cytotoxicity and the uptake of [^{3}H]TdR were assessed after 24 h. Aphidicolin at 1.5 μg/ml inhibited DNA synthesis by more than 99% when added to cultures containing either IL-2 (Fig. 6*A*) or IL-4 (Fig. 6*B*). It also inhibited approximately 80% of the IL-2 mediated activation of peCTL to cytotoxicity (Fig. 6*A*), and even more of the IL-4-mediated effect (Fig. 6*B*). The effect of aphidicolin was specific, in that no decrease in viability of cells was noted at 1.5 μg/ml, although at 5 μg/ml viability was reduced to 75%.

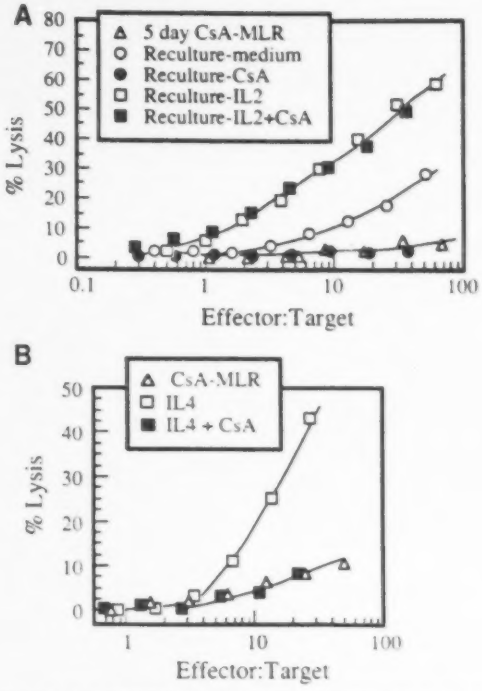

Figure 5. CsA inhibits the development of cytotoxicity in recultured peCTL induced by IL-4, but not by IL-2. *A*, MLR cultures were set up as in Figure 1, with CsA present at 300 ng/ml. On day 5, cells were washed and recultured for 48 h as follows: reculture-medium—fresh medium; reculture-CsA—300 ng/ml CsA; reculture-IL-2—20 U/ml IL-2; reculture-IL2 + CsA—300 ng/ml CsA plus 20 U/ml IL-2. Cytotoxicity was assayed on P815 target cells. The cytotoxicity on day 5, before reculture, is also given ("5 day CsA-MLR"). *B*, A primary CsA-MLR was set up as in *A*, with 300 ng/ml of CsA and 20 U/ml IL-2. On day 5, recovered cells were washed and recultured for 24 h with 4 ng/ml IL-4 or 4 ng/ml IL-4 plus 300 ng/ml CsA. The cytotoxic activity of untouched, original cultures was also measured on day 6 ("CsA-MLR").

Expression of serine esterase mRNA during reculture. A set of related, CTL-specific genes encoding serine esterases has recently been identified (28, 36). Expression of these genes is apparently restricted to cytotoxic T cells, as the corresponding mRNA have not been detected in resting thymocytes, B cells, macrophages or nonlymphoid cells. It has been postulated that serine esterases present in the granules of cytotoxic cells may play a role in CTL-mediated lysis (36). We used cDNA probes which encode two of these esterases, CCP1 (granzyme B_1 and CCP2 (granzyme C) to examine their expression during the development of cytotoxicity from peCTL cells harvested in a CsA-MLR. As shown in Figure 7, there was only a low level of the CCP1 and CCP2 mRNA in CsA-MLR cultures isolated on days 4 or 5. After reculture of 5 day CsA-MLR cells in IL-2, there was a rapid increase

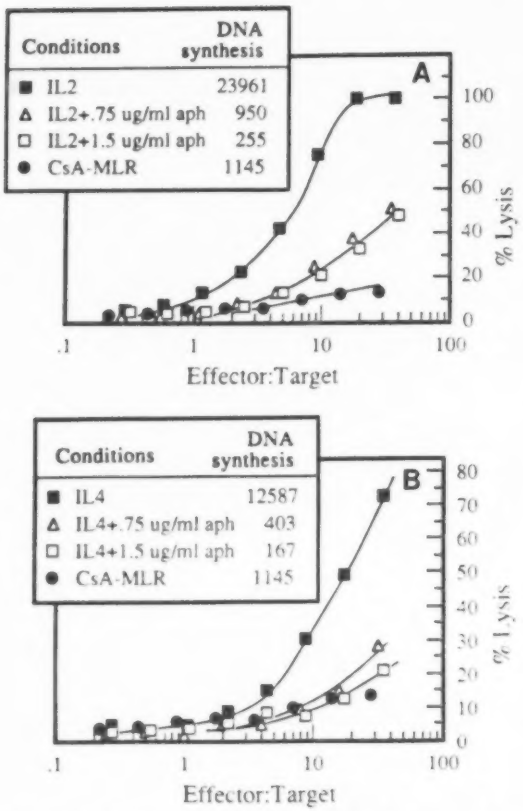

Figure 6. An inhibitor of DNA polymerase α-blocks most of the cytotoxicity induced by IL-2 or IL-4 in peCTL. MLR cultures were set up as in Figure 1, with 300 ng/ml CsA and 20 U/ml rhIL-2. After 5 days, viable cells were recovered by density gradient centrifugation, washed, and recultured for 24 h at 5×10^5 cells/ml with 20 U/ml of rhIL-2 (*A*) or 4 ng/ml IL-4 (*B*) and the indicated concentrations of aphidicolin. A portion of each culture was then set up in a 6-h proliferation assay, with the values of [^{3}H]TdR incorporation given as cpm/10^5 cells. The rest of the cells were washed and assayed for cytotoxicity on ^{51}Cr labeled P815 target cells. Viable cells from the 6-day primary CsA-MLR were separated by density gradient centrifugation before assay.

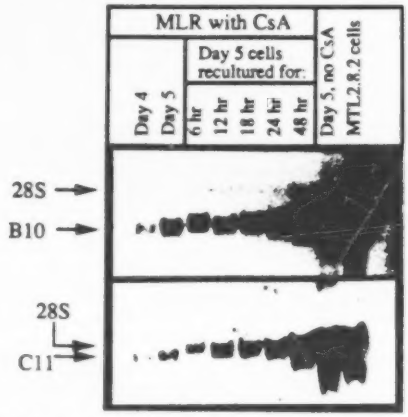

Figure 7. Two CTL-specific serine proteases are induced during the activation of peCTL to cytotoxicity. One-way MLR cultures were set up as in Figure 1, with 20 U/ml of rhIL-2 and, where indicated, 300 ng/ml of CsA. For the reculture groups, cells were washed and recultured on day 5 at 5×10^5/ml in 20 U/ml rhIL-2 for up to 48 h. Ten μg of total RNA was loaded per lane for Northern gel analysis. The blots were examined by ethidium staining for rRNA, and by an actin mRNA probe, and found analyses indicated, that as far as could be judged, the lanes all contained the same levels of RNA. The same blot was probed with the ^{32}P-labeled cDNA probes B10 and C11, which encode the serine proteases CCP2 and CCP1, respectively (36). Control lanes contained RNA from a 5-day MLR without CsA, and from MTL2.8.2 cells, from which B10 and C11 were originally cloned (28).

that approached the levels seen in cells from untreated MLR cultures, or in the CTL cell line MTL2.8.2 from which the genes were originally cloned. The pattern of expression of the serine protease genes correlated with the capacity of the cells to express specific cytotoxicity.

DISCUSSION

The conversion of precursors to CTL can be divided into stages by a number of experimental procedures. We have shown that CsA blocks development at the level of peCTL, which are noncytotoxic cells capable of rapid differentiation into the effector stage by exposure to IL-2 or IL-4. Although peCTL appear to develop in CsA-treated cultures in an IL-2-independent manner, we cannot exclude the possibility that a small, undetectable amount of IL-2 was present. IL-2-independent proliferation of the peCTL may also be postulated, as has been observed with CD8$^+$ T cells activated with anti-CD2 antibody (37). Ornithine has a somewhat similar effect to CsA in that CTL precursors are arrested at a precytolytic stage, but that stage of development is probably earlier than the peCTL stage we describe because ornithine does not inhibit the production of IL-2 and IL-3 (20).

The development of effector CTL from noncytotoxic peCTL in CsA-MLR cultures is probably limited simply by the inhibition of lymphokine production, because exposure to IL-2 rapidly leads to cytotoxicity (Fig. 2 and 3). However, the high level of CTL activity that can rapidly be generated by exposure to IL-2 (compare for example the level of cytotoxicity derived from recultured cells with that of control, non-CsA-treated cultures in Fig. 1*A*) suggests that a significant degree of clonal expansion has occurred at the peCTL stage.

The rapid consumption of rhIL-2 by MLR cultures, especially in the presence of CsA (Table I; Fig. 1*B*), means that IL-2 concentrations must be measured directly to conclude that it was, or was not, present at some point in the culture. Even 50 U/ml added on day 1 disappeared by day 5 in the presence of 250 ng/ml CsA (Table I). In earlier studies where IL-2 was added to CsA-MLR cultures (11, 30), consumption may have rapidly reduced its concentration, and conclusions regarding its effects are not simply interpretable.

A critically important result is that no cytotoxicity was generated in the continuous presence of IL-2 throughout the CsA-MLR (Fig. 1). Yet, when IL-2 was present only transiently at the onset of culture, or not at all, the addition of IL-2 on day 5 rapidly produced cytotoxicity (Fig. 3). It is evident that some kind of suppressive element is generated and maintained in the presence of CsA as long as IL-2 is present. A similar conclusion, based on somewhat different evidence, was reached previously by Bucy (9). Our results extend the earlier observations by showing that, although no cytotoxic activity is found in the CsA-inhibited cultures, an activated peCTL state is achieved. If IL-2 is allowed to disappear, as it does in the CsA-MLR left alone, the peCTL formed are rapidly con-

verted to CTL by the addition of IL-2 at later times. We also conclude that the suppressor elements generated in the presence of CsA and IL-2 do not inhibit by consuming all of the IL-2—the monitored continuous presence of IL-2 in Figure 1 shows that suppression and IL-2 are both present.

The development of suppressive cells in the presence of CsA in human MLR cultures has been described (38). In that work, the addition of exogenous IL-2 was not necessary. Those studies used 8-day human MLR cultures, and assayed them for suppressor activity in second cultures over 3 days' further culture. Much higher levels of CsA were needed to obtain Ag-specific suppression, and it was apparently dependent on the particular ratios of suppressor to responder cells used in the second cultures.

The effects of CsA in the MLR cultures can be understood in terms of the next model. CsA inhibits the production of lymphokines that are essential for the generation of cytotoxic cells (probably IL-2, perhaps IL-4, possibly others), but permits the development of activated precursors of CTL, the peCTL, by proliferation as well as differentiation. However, if IL-2 is present throughout the CsA-MLR, no cytotoxic cells develop, because IL-2 and CsA together induce a persistent suppression. This is consistent with earlier results showing that Ts cells, although inhibited in CsA-treated MLR cultures, do develop if exogenous IL-2 is provided (9).

What is not yet clear is whether CsA directly inhibits the activation of primary pCTL. Preliminary results suggest that it does not, at least at the levels used here. Thus, we could detect peCTL in cultures maintained with CsA and IL-2, suggesting that activation of pCTL to peCTL was occurring, even though CTL were not developing because of Ts cells. However, this area requires more refined analysis. We have previously described a direct effect of CsA on cloned, Ag-dependent CTL, in which the Ag-mediated reactivation of quiescent, noncytotoxic cells to proliferate and kill was blocked by CsA, but the IL-2 "second signal" was not (13). Interpretation of studies on CsA-mediated inhibition is complicated by the differences seen with different levels of CsA, with higher concentrations having qualitatively different, although still relatively specific, effects compared to lower levels. For example, IL-2 production can be 50% inhibited by 10 ng/ml CsA (7), whereas levels of about 100 ng/ml are required to block the reactivation of quiescent CTL clones (13). In a more complex example, IL-2 reversed the CsA-mediated inhibition of CTL generation at levels of CsA less than 100 ng/ml, but not at higher concentrations (8).

We have shown that peCTL can develop in the absence of exogenous or detectable IL-2 (Figs. 4 and 5*A*) and can be induced to differentiate to CTL by the addition of rhIL-2. IL-2 appears to be sufficient for the differentiation of peCTL into allospecific CTL but the possibility that this effect is mediated indirectly cannot be excluded. Earlier results showing that IL-4 can induce cytotoxicity (29, 30) did not demonstrate an absolute requirement for IL-4. Anti-IL-4 had little effect on the induction of cytotoxicity by IL-2 in our experiments, but did reduce the proliferative response, suggesting that IL-4 is produced during the reculture period. These results suggest that its main effect is in modulating the proliferative response, in accordance with our previous observations (see footnote 4). It is not clear that IL-4 was affecting the peCTL directly, because CsA inhibited the increase in cytotoxicity induced by it (Fig. 5*B*). Instead, IL-4 may have been acting to induce the production of IL-2 or another lymphokine. A cytotoxic differentiation factor for CTL generation, which is probably IL-6, has recently been described (39). The model of CsA-mediated arrest at the stage of peCTL should be helpful for the molecular analysis of the steps involved in the conversion of precursors to CTL.

Acknowledgments. We are grateful to Dr. Kim Bottomly (Yale University) for the anti-IL-4 antibody 11B11, and to Dr. Gerard F. Murphy, Sandoz Canada Inc. for CsA.

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